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Polarización de macrófagos: efecto de las inmunoglobulinas y de la serotonina

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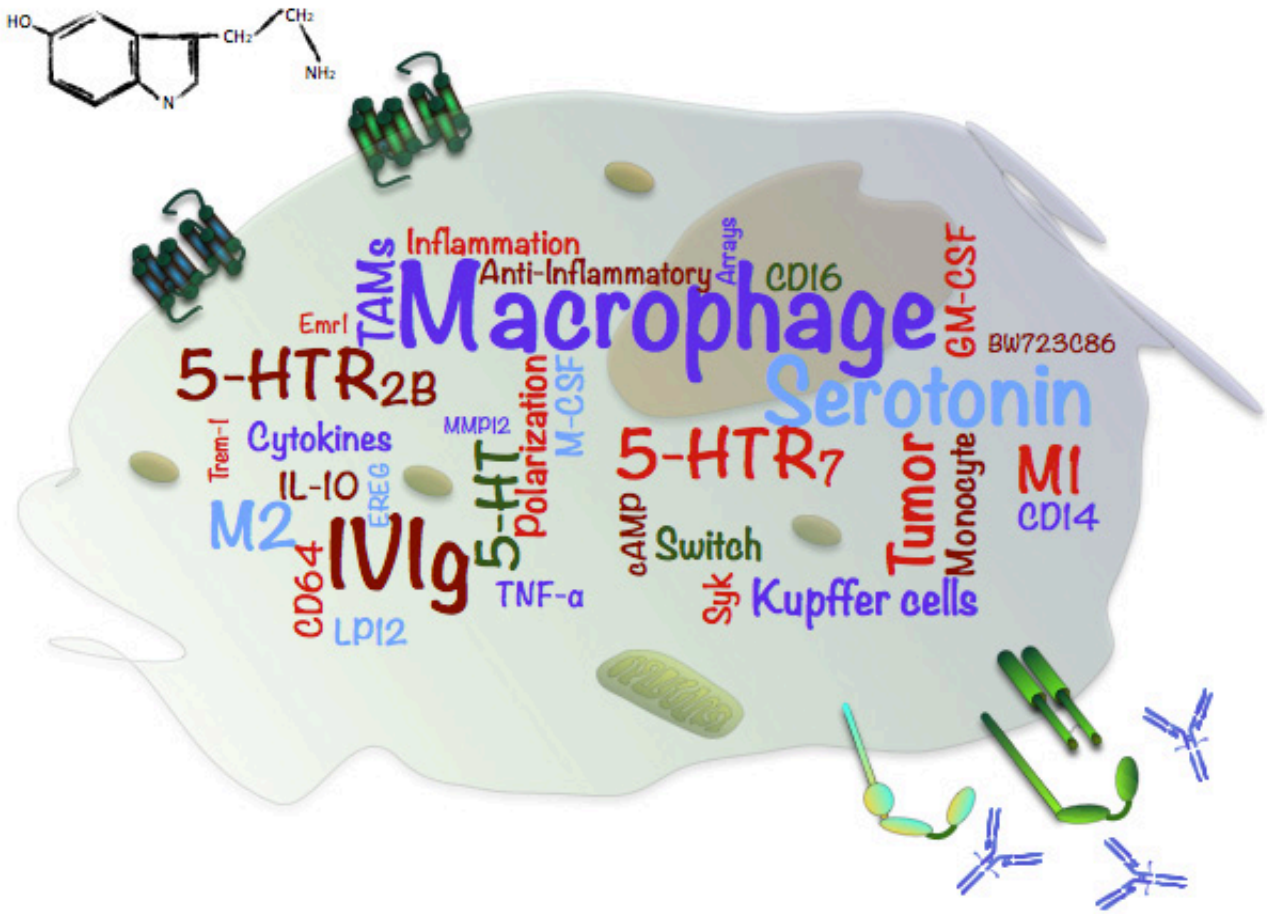

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Este trabajo ha sido realizado por Mateo de las Casas Engel para optar al grado de Doctor, en el Centro de Investigaciones Biológicas de Madrid (CSIC), bajo la dirección del Dr. Ángel L. Corbí López.

Fdo. Ángel L. Corbí Lopez.

Abbreviations

5-HT Serotonin

ATM Adipose Tissue Macrophages

cAMP Cyclic Adenosine Monophosphate

CNS Central Nervous System

CCL Chemokine (C-C motif) Ligand 2

CXCL Chemokine (C-X-C motif) Ligand

DC Dendritic Cell

EGF Epidermal Growth Factor

Fab Fragment Antigen-Binding

FC Fragment Crystallizable region

FCγR FC-gamma Receptor

FDA Food and Drug Administration

GM-CSF Granulocyte Macrophage Colony-Stimulating-Factor

IFN Interferon

Ig Immunoglobulin

IL Interleukin

IVIg Intravenous Immunoglobulin

M-CSF Macrophage Colony-Stimulating-Factor

MDDC Monocyte Derived Dendritic Cells

LPS Lipopolysaccaride

PAH Pulmonar Arterial Hypertension

TAMs Tumor Associated Macrophage

TGF Transforming Growth Factor

Th T-helper

TNF Tumor Necrosis factor

TPH Tryptophan Hydroxylase

VHD Valvular Heart Disease

ABSTRACT

Introduction

Although GM-CSF and M-CSF contribute to both cell survival and proliferation, they exert distinct actions during macrophage development. The lack of M-CSF alters the development of various macrophage populations, whereas GM-CSF-deficient mice only exhibit altered maturation of alveolar macrophages. Along the same line, both cytokines promote the in vitro differentiation of macrophages with distinct morphology, pathogen susceptibility and inflammatory function. GM-CSF drives the generation of monocyte-derived macrophages that produce pro-inflammatory cytokines in response to LPS such as IL-12p40 or TNFα and display high antigen-presenting and tumoricidal capacity (M1-polarized macrophages). On the other hand, M-CSF yields macrophages that release IL-10 in response to pathogens and exhibit high phagocytic and pro-tumoral activity (M2-polarized macrophages). Accordingly, and based on their respective cytokine and gene expression profiles, human macrophages generated in the presence of GM-CSF or M-CSF are considered as pro-inflammatory and anti-inflammatory, respectively.

Serotonin (5-hydroxytryptamine, 5-HT) is synthesized from L-tryptophan via a rate-limiting reaction catalyzed by two tryptophan hydroxylases encoded by genes with a distinct pattern of expression (TPH1 in periphery, TPH2 in brain). Outside the central nervous system, 5-HT is synthesized and released into the circulation by enterochromafin cells, and is rapidly taken up and stored by platelets and, to a lesser extent, lymphocytes, monocytes and macrophages. Serotonin signals through seven different receptors ($5\text{-HTR}_{1\text{-}7}$), six of which belong to the G protein-coupled superfamily of receptors (5-HTR_1, 5-HTR_2, 5-HTR_4, 5-HTR_5, 5-HTR_6, 5-RHT_7). Outside the central nervous system, 5-HT play an important role as a growth factor and as a regulator of inflammation and tissue regeneration and repair, modulating cytokine production in a immune cell type-dependent manner.

Intravenous immunoglobulin (IVIg) is a preparation of polyclonal and polyspecific immunoglobulins (mostly IgG) derived from the plasma of thousands of healthy donors. IVIg exerts a potent immunomodulatory action in immunodeficiency syndromes, autoimmune diseases and infectious processes. Besides, previous studies have shown that IVIg impairs the metastatic spread of carcinomas in mice, and contributes to tumor regression in a number of cancer patients, leading to the proposal of IVIg as a potential anti-metastatic drug. However, the molecular basis for the IVIg immunomodulatory action and anti-tumoral effect remains to be completely clarified.

Results

Considering the importance of macrophage polarization plasticity for inflammatory responses and tissue repair, we evaluated whether 5-HT modulates human macrophage polarization. 5-HT inhibited the LPS-induced release of pro-inflammatory cytokines without affecting IL-10 production, upregulated the expression of M2 polarization-associated genes (*SERPINB2, THBS1, STAB1, COL23A1*) and reduced the expression of M1-associated genes (*INHBA, CCR2, MMP12, SERPINE1, CD1B, ALDH1A2*). Whereas only 5-HTR_7 mediated the inhibitory action of 5-HT on the release of proinflammatory cytokines, both 5-HTR_{2B} and 5-HTR_7 receptors mediated the pro-M2 skewing effect of 5-HT. In fact, blockade of both receptors during in vitro monocyte-to-macrophage differentiation preferentially modulated the acquisition of M2 polarization markers. 5-HTR_{2B} and 5-HTR_7 were found to be preferentially expressed by anti-inflammatory M2 (M-CSF) macrophages and, in the case of 5-HTR_{2B}, it was also detected in vivo in liver Kupffer cells and in Tumor-Associated Macrophages.

Next, we undertook the identification of the 5HT-dependent gene expression profile in human macrophages. We report that the 5-HTR_7-cAMP-PKA axis is responsible for the 5-HT-dependent expression of genes encoding growth factors (*EREG*), growth factor receptors (*MET*), cytokines (*IL1B*) and cell surface molecules involved in macrophage activation (*TREM1*), all of which might contribute to the growth-promoting and immunomodulatory functions of 5-HT. In addition, we have determined that 5-HT, via ligation of 5-HTR_{2B}, regulates the expression of type I IFN-responsive genes (*CXCL10, IFI2, IFIT3, TNFSF18*), thus defining a novel link between 5-HT and the expression of genes that govern antiviral responses by human macrophages. Therefore, 5-HT modulates macrophage polarization and contributes to the maintenance of an anti-inflammatory state via 5-HTR_{2B} and 5-HTR_7, whose identification as functionally-relevant markers for anti-inflammatory/homeostatic human M2 macrophages suggests their potential therapeutic value in inflammatory pathologies.

The balance between polarization states contributes to inflammation resolution, and its alteration underlies inflammatory pathologies and cancer. Since Intravenous immunoglobulin (IVIg) exerts immunomodulatory actions and limits tumor growth and metastasis, we hypothesized that IVIg immunomodulatory activity might rely on its ability to alter macrophage polarization. We now demonstrate that IVIg abrogates GM-CSF- or M-CSF-driven monocyte polarization, and induces a CD16- and Syk-dependent transcriptional and functional

polarization switch on monocyte-derived human macrophages. Likewise, the IVIg-promoted polarization switch is inhibited or prevented in *Cd16*$^{-/-}$ or *Fcer1g*$^{-/-}$ mouse macrophages. Besides, IVIg alters the polarization of tumor-associated CD11b^{+} cells in tumor mouse models, where it also impairs tumor progression in a Cd16- and Fcer1g-dependent manner. Therefore, IVIg halts monocyte polarization and exerts a potent M1 re-polarization ability on M2 macrophages. These observations link the anti-tumoral and macrophage re-polarization abilities of IVIg, and reveal that the IVIg immunomodulatory effects are dependent on the polarization state of the responding macrophages. This property of IVIg could be therapeutically useful in pathologies like cancer, where immunogenic and pro-inflammatory macrophage functions need to be promoted.

Conclusions

We here demonstrate that 5-HT-receptors 5-HTR$_{2B}$ and 5-HTR$_{7}$ are preferentially expressed by *in vitro* M-CSF-derived macrophages, and that 5-HTR$_{2B}$ is expressed by human macrophages *in vivo*, including Kupffer cells and Tumor Associated Macrophages (TAM). The ability of 5-HT to modulate macrophage polarization towards to an M2-like phenotype is mediated by 5-HTR$_{2B}$ and 5-HTR$_{7}$, while its ability to inhibit the LPS-induced secretion of pro-inflammatory cytokines is via ligation of 5-HTR$_{7}$. The gene expression profile observed in macrophages treated with 5-HT is dependent on the 5-HTR$_{7}$/cAMP/PKA axis, while the activation of 5-HTR$_{2B}$ by BW723C86 promotes the expression of type I IFN-regulated genes.

In the case of IVIg, we demonstrate that IVIg reduces the LPS-induced secretion of pro-inflammatory cytokines from M1 macrophages without significantly affecting the transcriptomic polarization state. Meanwhile, on M2 macrophages, IVIg switches the phenotypic and functional polarization of human and murine M2 macrophages towards the acquisition of an M1-like state. This switch is partly dependent on CD16 on human macrophages, and Fc receptor-mediated in murine macrophages. Since the M2-to-M1 switch might have an important role on inhibiting tumor progression, we demonstrate that IVIg treatment triggers an increase in pro-inflammatory gene markers (Such as *Inhba, Ccr7, Egln3, Cd11c, Nos2*) in intra-tumoral macrophages, as well as a change in the basal level of cytokines (such as Tnfa and Ccl2) in the peripheral blood of tumor-bearing animals. We further demonstrate that IVIg exerts an anti-tumoral activity in murine models of cancer, an effect that is dependent on macrophages.

RESUMEN

Introducción

Tanto el GM-CSF como el M-CSF contribuyen a la supervivencia y a la proliferación celular. Sin embargo, ejercen acciones diferentes durante la polarización de los macrófagos. La ausencia de M-CSF altera el desarrollo de diversas poblaciones de macrófagos, mientras que en los ratones deficientes en GM-CSF sólo se ve afectada la maduración de los macrófagos alveolares. Ambas citoquinas promueven la diferenciación in vitro de macrófagos dando lugar a dos poblaciones con una morfología, respuesta a patógenos y función inflamatoria distintas. Mientras que el GM-CSF promueve la generación de macrófagos "clásicos", o M1 (que se caracterizan por ser productores de citoquinas pro-inflamatorias en respuesta a LPS, tales como IL - 12p40 o TNFα, además de por una alta capacidad presentadora de antígeno y actividad tumoricida), el M-CSF produce macrófagos "alternativos", o M2, que liberan IL-10 en respuesta a agentes patógenos, poseen una alta capacidad fagocítica y actividad pro-tumoral. De esta manera, y en base a sus respectivos perfiles de producción de citoquinas y la diferente expresión génica, los macrófagos humanos generados en presencia de GM-CSF (M1) o M-CSF (M2) se consideran pro- o anti-inflamatorios, respectivamente.

La serotonina (5-hidroxitriptamina, 5-HT) se sintetiza a partir de L-triptófano a través de una reacción catalizada por dos triptófano-hidroxilasas (TPH) que se encuentran diferencialmente expresadas en el organismo (TPH1 en periferia, TPH2 en el cerebro). Fuera del sistema nervioso central, 5-HT se sintetiza y libera en la circulación por células enterocromafinas del intestino. Posteriormente sufre una rápida recaptura por las plaquetas, donde pasará a ser almacenada. En menor medida, linfocitos, monocitos y macrófagos también pueden almacenar y captar la serotonina circulante. Existen siete tipos de receptores de serotonina (5-HTR_{1-7}), seis de los cuales pertenecen a la superfamilia de receptores acoplados a proteína G (5-HTR_1, 5-HTR_2, 5-HTR_4, 5-HTR_5, 5-HTR_6, 5-RHT_7). Fuera del sistema nervioso central, 5-HT juega un papel importante como factor de crecimiento, así como de regeneración y reparación de tejidos. La 5-HT es capaz de actuar como un regulador de la inflamación, modulando la producción de citoquinas en células del sistema inmune de una forma dependiente del tipo celular.

Las inmunoglobulinas intravenosas (IVIg) constituyen una preparación de inmunoglobulinas policlonales y poliespecíficas (principalmente IgG) derivada a partir del plasma de miles de donantes sanos. IVIg ejerce una acción inmunomoduladora potente en síndromes de inmunodeficiencia, enfermedades autoinmunes y procesos

infecciosos. Además, estudios anteriores han demostrado que IVIg inhibe la diseminación metastásica en carcinomas de ratón, y contribuye a la regresión tumoral en pacientes con cáncer, lo que ha llevado a proponer las inmunoglobulinas como un fármaco potencialmente anti-metastásico. A pesar de esto, las bases moleculares de la acción inmunomoduladora, así como el mecanismo de su efecto anti-tumoral, aún no se han aclarado por completo.

Resultados

Dada la importancia de la plasticidad de polarización de los macrófagos para las respuestas inflamatorias, así como para la reparación de tejidos, evaluamos si 5-HT modula la polarización de macrófagos humanos. En este trabajo se muestra la presencia de los receptores 5-HTR_{2B} y 5-HTR_{7} diferencialmente expresados en macrófagos M2 generados in vitro. Además, 5-HTR_{2B} fue detectado in vivo en células de Kupffer del hígado y en los macrófagos asociados a tumores (TAM).

También observamos que la 5-HT inhibió la liberación de citoquinas pro-inflamatorias (IL-12p40 y TNFα) en respuesta a LPS -sin afectar a la producción de IL-10- a través de la activación de 5-HTR_{7}. Además, indujo un aumento en la expresión de genes asociados a polarización M2 (*SERPINB2, THBS1, STAB1, COL23A1*) y una reducción de expresión de genes asociados a estado de polarización M1 (*INHBA, CCR2, MMP12, SERPINE1, CD1b, ALDH1A2*) mediada por ambos receptores 5-HTR_{2B} y 5-HTR_{7}. De hecho, el bloqueo farmacológico de ambos receptores durante la diferenciación in vitro de monocitos a macrófagos en presencia de M-CSF provocó una alteración en la adquisición de genes marcadores de polarización M2.

A continuación, se analizó el perfil de la expresión génica dependiente de 5-HT en los macrófagos humanos. En este informe describimos como el eje 5-HTR_{7} -AMPc-PKA es mayoritariamente responsable de la expresión de genes dependientes de 5-HT entre los que se incluyen el factor de crecimiento (*EREG*) , receptores del factor de crecimiento de hepatocitos (*MET*) , citoquinas (*IL1B*) y moléculas de la superficie celular involucradas en la activación de macrófagos (*TREM1*), que podrían contribuir a la promoción del crecimiento y las funciones inmunomoduladoras de 5-HT. Además, hemos determinado que 5-HT, a través del receptor 5-HTR2B, regula la expresión de los genes sensibles a IFN de tipo I (*CXCL10 , IFI2 , IFIT3 , TNFSF18*) , definiendo de este modo un nuevo enlace entre la serotonina y la inducción de genes encargados de promover respuestas antivirales en los macrófagos humanos. Por lo tanto, 5-HT modula la polarización de macrófagos y contribuye

al mantenimiento de un estado anti- inflamatorio a través de 5-HTR_{2B} y 5-HTR_{7}. La identificación de estos receptores como marcadores funcionalmente relevantes para macrófagos humanos M2 (anti-inflamatorios/ homeostáticos) les confiere un potencial valor terapéutico en patologías inflamatorias.

El equilibrio entre los estados de polarización contribuye a la resolución de la inflamación, y alteraciones en este equiibrio pueden desembocar en patologías inflamatorias o cáncer. Puesto que IVIg ejerce acciones inmunomoduladoras y limita el crecimiento tumoral y las metástasis, nuestra hipótesis fue que dichas acciones podrían deberse a cambios en el estado de polarización de los macrófagos. En este trabajo demostramos cómo IVIg es capaz de inhibir ciertas actividades de los macrófagos M1, (como la secreción de citoquinas inflamatorias), mientras que en macrófagos M2, IVIg produce un cambio de polarización de un estado anti-inflamatorio (M2) a uno pro-inflamatorio (M1), tanto a nivel funcional como génico. Esta re-polarización de macrófagos es mediada parcialmente en humanos por CD16 y SyK, mientras que en macrófagos de ratón es dependiente también de CD-16 y de *Fcer1g*. Además, IVIg inhibe el crecimiento tumoral en modelos de ratón y altera la polarización de las células $CD11b^{+}$ asociadas a tumores, de manera dependiente de macrófagos y los receptores CD16 /*Fcer1g*.

Conclusiones

Con este trabajo demostramos que los receptores de serotonina 5-HTR_{2B} y 5-HTR_{7} se expresan preferencialmente en macrófagos generados in vitro en presencia de M-CSF, y que el receptor 5-HTR_{2B} se encuentra además en macrófagos humanos in vivo, incluyendo las células de Kupffer y macrófagos asociados a tumores (TAM).

La capacidad de 5-HT para modular la polarización de macrófagos hacia un fenotipo M2 está mediada por 5-HTR_{2B} y 5-HTR_{7}, mientras que su capacidad para inhibir la secreción de citoquinas pro-inflamatorias inducida por LPS está regulada por 5-HTR_{7} . Además, el perfil de expresión génica observado en los macrófagos tratados con 5-HT es principalmente dependiente del eje 5-HTR_{7}/cAMP/PKA, mientras que la activación de 5-HTR_{2B} por BW723C86 promueve la expresión de genes regulados de tipo I-IFN.

En el caso de IVIg, demostramos que reduce la secreción de citoquinas pro-inflamatorias inducida por LPS en los macrófagos M1, sin afectar significativamente el estado de polarización transcriptómica. Sin embargo, en

los macrófagos M2, IVIg cambia las características fenotípicas y funcionales de los macrófagos (M2 humanos y murinos) hacia la adquisición de un estado tipo M1. Esta re-polarización es parcialmente dependiente de CD16 en macrófagos humanos, y de CD16 y otros receptores asociados a Fcer1g en los macrófagos de ratón. La re-polarización M2-a-M1 podría tener un papel importante en la inhibición de la progresión tumoral. De hecho, nuestros resultados demuestran que el tratamiento con IVIg provoca un aumento de marcadores génicos asociados a macrófagos M1, (pro-inflamatorios y anti-tumorales), (tales como *INHBA, CCR7, EGLN3, CD11c, NOS2*) en macrófagos intra-tumorales , así como un cambio en el nivel basal de citoquinas (tales como TNFa y Ccl2) en la sangre periférica de los animales en los que se les indujo un tumor. Además, demostramos que IVIg ejerce una actividad anti-tumoral en modelos de ratón de cáncer, un efecto que depende casi exclusivamente de los macrófagos.

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INTRODUCTION

1. MACROPHAGES

Macrophages are bone marrow-derived cells and, together with neutrophils, constitute the first line of resistance against pathogens, for which they provide a fast and unspecific response (innate immunity). Besides, and together with dendritic cells, macrophages are essential for the coordinated orchestration of innate and adaptive immune responses. Since the latest 1800, when Metchnikoff described the existence of phagocytes (1), numerous studies have addressed their developmental origin as well as their phenotypic and functional diversity (fig.1). Although still a matter of debate, macrophages can differentiate from peripheral blood monocytes and, once within tissues, become effector cells whose huge functional plasticity allows them to play essential roles in physio-pathological processes as diverse as pathogen clearance, tissue repair, angiogenesis and tumour progression and metastasis. (2). The functional plasticity of macrophages arises from their ability to respond to endogenous and non-self stimuli while adapting to the surrounding tissue environment (3-6), and explains both the existence of tissue-specific macrophages (microglia, osteoclasts, Kupffer cells, …) and the wide variety (continuum) of macrophage activation states (polarization).

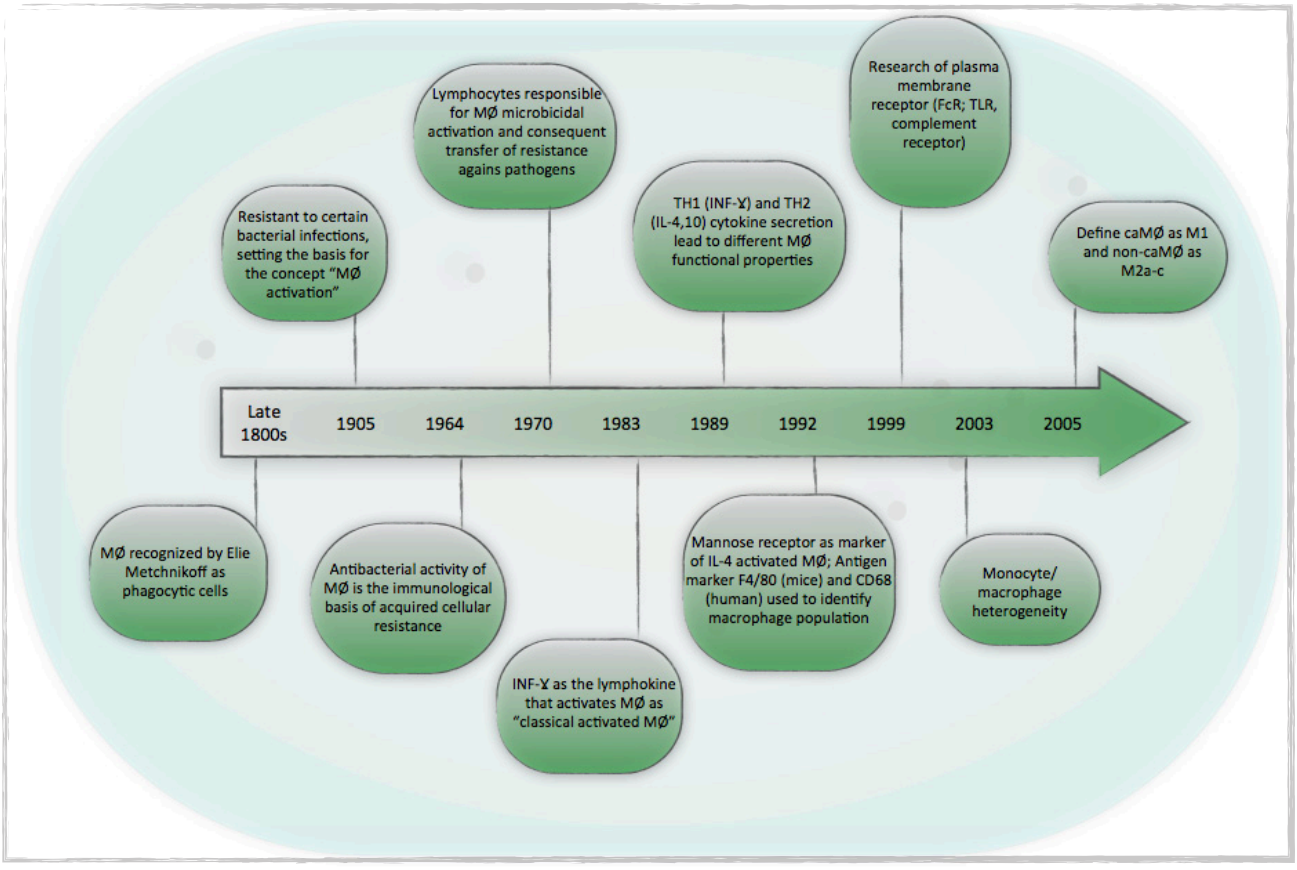


Figure 1. Timeline: advances on macrophage biological research. Figure depicts some of the most relevant findings in the field.

1.1 MACROPHAGE POLARIZATION

In response to microbe-derived factors, cytokines from Th1 cells (e.g. IFNγ) or other cytokines such as GM-CSF or TNFα, macrophages acquire pro-inflammatory, bactericidal, tumor suppressive and immunogenic activities, in a process commonly referred to as "classic" or M1 polarization, and whose hallmark is the ability to release large amounts of IL-12/IL-23, reactive nitrogen and oxygen intermediates, and expression

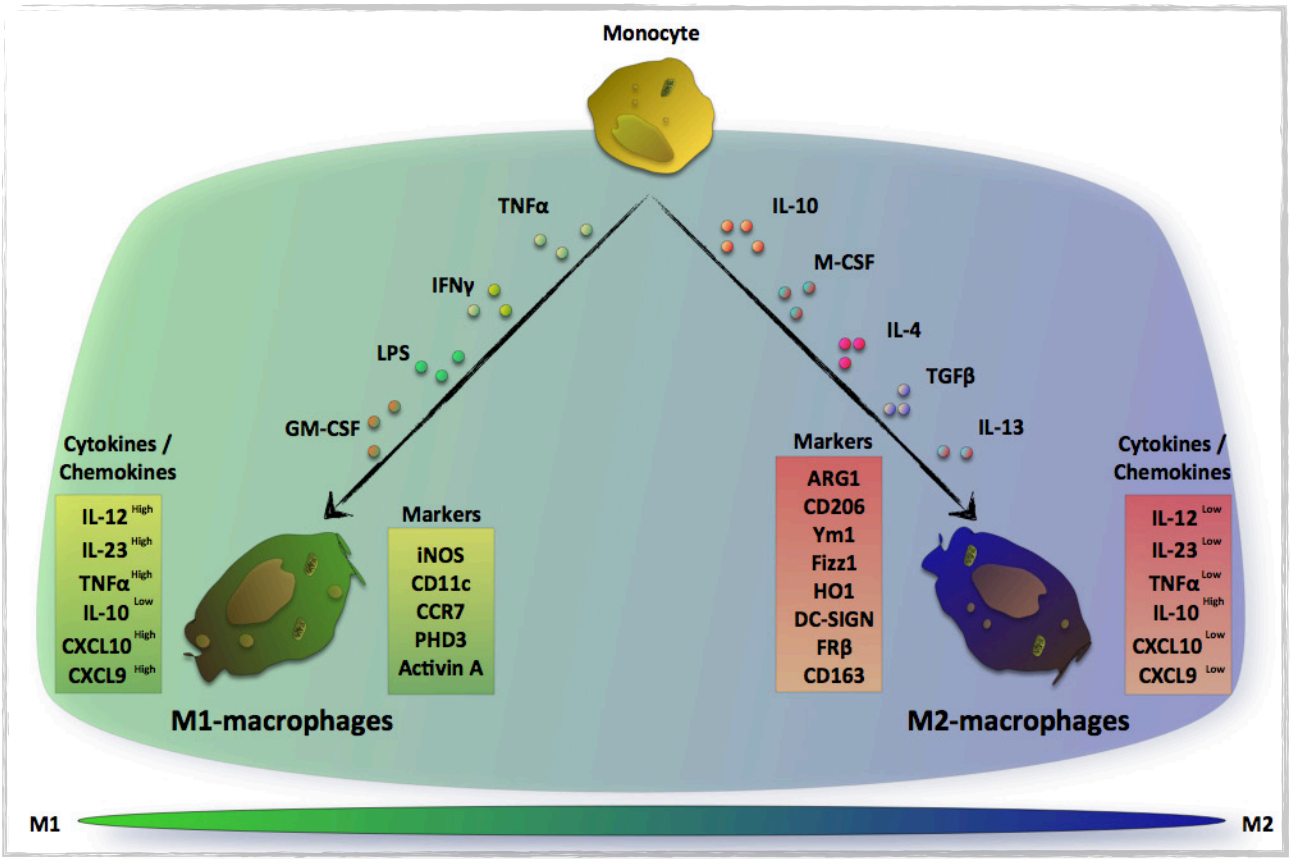


Figure 2. Different cytokines that induce macrophage polarization.

of Th1-cell attracting chemokines (2, 4) (fig.2). M1 macrophages also drive the polarization and recruitment of Th1 cells through the expression of cytokines and chemokines like IL-12, CXCL9 and CXCL10, thereby amplifying Type-1 immune responses (2). Conversely, Th2-derived cytokines like IL-4, IL-13, IL-10, TGFβ or M-CSF, as well as glucocorticoids, promote the acquisition of anti-inflammatory, scavenging, tumor-promoting, tissue repair and pro-angiogenic functions, all of which are grouped under the terms "alternative" or M2 polarization, that endows them with the ability to produce high levels of IL-10 (2, 5-8) (fig.2). Therefore, the classical M1 and M2 macrophage polarization states are just two extremes of a wide range of functionally distinct macrophages activation states (3-6) (fig.3).

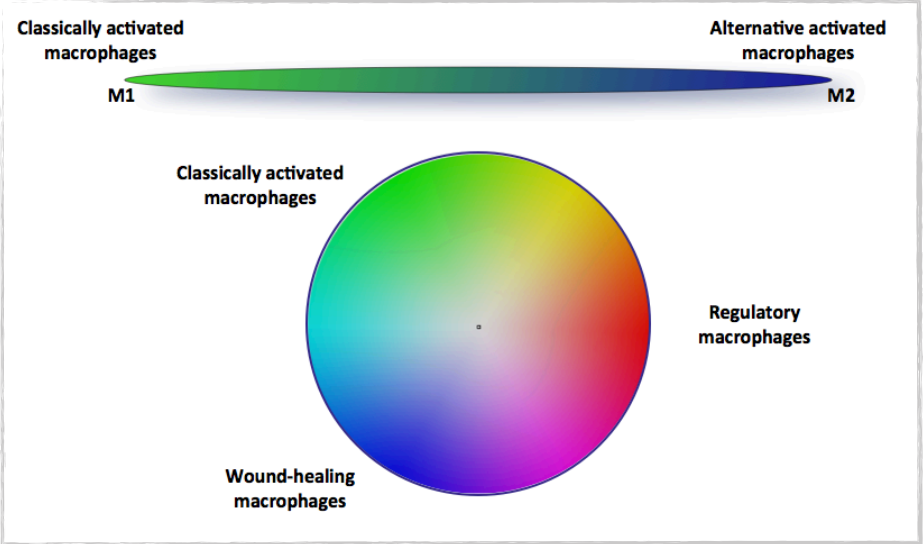


Figure 3. Colour wheel of macrophage activation. Adapted from Mossser and Edwars (3). On the top, linear scale of the two macrophage designations diferentation M1 and M2. On the bottom, the color wheel that shows the continum of macrophages polarization.

1.2 MACROPHAGES IN HEALTH AND DISSEASE

1.2.1 MACROPHAGES IN HOMEOSTASIS

Under homeostatic *in vivo* conditions, tissue-resident macrophages also exhibit a wide variety of polarization states, which are ultimately determined by the extracellular environment and the surrounding cell types. Thus, macrophages exhibit tissue-specific phenotypes and functions under homeostatic conditions. As representative examples, bone macrophages (osteoclasts) display potent bone-degrading functions, brain macrophages (microglia) (9) contribute to development of neural circuitry and modulation of angiogenesis and fluid balance in the brain (10), and liver macrophages (Kupffer cells) are primarily specialized in scavenging (11). Due to their localization in liver sinusoids, Kupffer cells come in contact with antigens absorbed via the gastrointestinal tract and, therefore, play a crucial role in identifying and detoxifying bacteria, endotoxins (12), cell debris, apoptotic cells and immune complexes as well as toxic agents such as ethanol (13).

Regarding basal polarization, the existence of IL-10-producing (M2-polarized) macrophages has already been demonstrated in lungs (14) and gut (15, 16), tissues that are continuously exposed to exogenous and potentially damaging substances. Gut macrophages function in host defence through the recognition, phagocytosis, and killing of microorganisms (17, 18), and display a weak pro-inflammatory cytokine profile. The same applies for lung macrophages, that constitutively secrete IL-10 and whose ability for pro-inflammatory cytokine production is low (14). Other tissues also appear to preferentially contain M2-skewed macrophages in homeostasis. Brain

microglia cells are dependent on M-CSFR signalling and also present an M2-like phenotype (9). In the case of liver, most Kupffer cells (80–90% of tissue macrophages of the body) (19) are also M-CSF-dependent (20), thus explaining their M2-skewed polarization state. Peritoneal fluid also contains large amounts of M-CSF (21), that ultimately results in peritoneal macrophages acquiring an M2-like phenotype (21, 22) characterized by a low capacity for T-cell activation and high IL-10 secretion levels after stimulation (22). Taken together, these observations indicate that macrophages display M2-associated effectors functions in various tissues under basal conditions, implying that the control of macrophage polarization is of fundamental importance for tissue homeostasis.

1.2.2 MACROPHAGES IN INFLAMMATION, RESOLUTION AND TISSUE REPAIR

It is along an inflammatory response where the physio-pathological importance of fine-tuning macrophage polarization is most easily understood. Thus, M1-polarized macrophages predominate at the initial stages of an inflammatory response, when cytotoxic and tissue-damaging activities are more robust. Later, M2-type macrophages increase in number, as a means to promote resolution of inflammation (by limiting Th1-dependent responses and promoting Th2-cell recruitment and amplifying Th2-responses) (23). Therefore, a critical issue for inflammation resolution is a change in the polarization state ("polarization switch") of the macrophages that are recruited towards inflamed tissues. Such a switch was elegantly demonstrated in the case of peritoneal infection by *L. monocytogenes*, where the initial influx of pro-inflammatory M1 monocytes is followed (after 6-8 hours) by a potent increase in "resolving" M2 monocytes (24). In fact, and in the resolution-phase, macrophages express a unique mixed M1/M2 phenotype, with cAMP being essential to restrain M1 activation (25). However, it remains to be determined whether pro-inflammatory macrophages within inflamed tissues are later converted into M2 macrophages. In this regard, it is generally accepted that such an M1-to-M2 switch takes place during inflammation, and that the capture of apoptotic cells is the primary stimulus for such a polarization switch. This idea has gained considerable support after the studies of Brüne´s group, who demonstrated that apoptotic cell-derived Sphingosine-1-Phosphate (S1P) triggers the expression of Heme Oxygenase 1 (HO1) and pushes macrophages towards the M2/anti-inflammatory polarization state (26). Regardless of the polarization switch-triggering event, the sequential occurrence of both polarization states is required for adequate resolution of inflammation and return to tissue homeostasis. Some representative examples are commented below.

In animal models of ischemic heart disease, the M1-to-M2 dynamic change in macrophage recruitment has been observed at an early inflammatory phase. After heart ischemia, macrophages primarily exhibit a "classical activation" (M1) phenotype, exemplified by their high expression of TNF-α, whereas their phenotype shifts into an "alternatively activated" phenotype (M2) (high Arginase 1 and 2 expression) during the transition from inflammation to scar tissue formation (27). Similarly, after spinal cord injury, activated microglia rapidly release pro-inflammatory cytokines, which contribute to the influx of neutrophils and macrophages from the circulation (28). However, the resolution phase is characterized by the influx of macrophages that exhibit anti-inflammatory and tissue-repair properties (M2), and whose injection promotes full recovery (29).

All current evidences indicate that the misbalance of the M1/M2 polarization equilibrium, or an inadequate switch in the macrophage polarization state in inflamed tissues, invariably leads to chronic inflammatory pathologies that include tumor development, autoimmune diseases (multiple sclerosis), modulation of T cell–mediated nervous system autoimmune disease (30), fat mass development, obesity-associated cardiovascular pathologies and insulin resistance (31-33). Human chronic venous ulcers (CVUs) represent a good example where macrophages fail to resolve a chronic inflammatory condition probably because of the failure to in the M1-to-M2 switch (34). In CVUs, iron overload appears to sustain M1 polarization, thus leading to ROS-mediated DNA damage, fibroblast cellular senescence and defective tissue repair (34). Obesity-associated insulin resistance, diabetes and metabolic syndrome are also sustained by a chronic subclinical inflammation. In obesity, adipocytes release mediators such as CCL2, TNFα or free fatty acids that promote the recruitment and subsequent M1-like activation of Adipose Tissue Macrophages (ATM) (35). The activation of these macrophages by inflammatory cytokines and saturated fatty acids provokes the inhibition of the insulin signalling pathway by JNK, IKK and IRS, the activation of AP-1 and NF-κB (further increasing pro-inflammatory cytokine secretion) and, consequently, leads to an insulin resistance(31, 36). ATMs from obese mice and human exhibit an M1-like profile, with up-regulation of TNFα and Nos2. In contrast, normal ATMs express high levels of M2-associated genes, including IL-10 and Arg1(32).

Tumor progression probably constitutes the paradigmatic example of the pathological consequences of de-regulated macrophage polarization (37). Macrophages play a key role in cancer-related inflammation, and their presence usually correlates with a poor outcome. The link between infiltration by Tumor Associated Macrophages (TAMs) and a bad prognosis extends to the case of Classic Hodgkin's Lymphoma (38), colon cancer (39),

breast cancer (40), hepatocellular carcinoma (41), melanoma (42) and many other tumors. In fact, the tumor environment has a key role in determining the immune suppressive capacity of TAMs (fig.4). Monocytes from peripheral blood are recruited into the tumor by M-CSF and chemokines like CCL2 and CXCL12. At the early stages of tumor-promoted inflammation, macrophages secrete pro-inflammatory cytokines (TNFα, IL-12, IL-1 and IL-6), which might reduce tumour growth and progression (43, 44). However, this anti-tumoral behaviour turns immunosuppressive and pro-tumoral as cancer progresses (45). This is so because tumor and stromal cells contribute to the modulation of macrophage effector functions (polarization) by secreting M-CSF, IL-10, IL-6 and VEGF (46), all of which drive the acquisition of M2-associated properties. Tumor-recruited myeloid cells are also responsible for the expression of M2-promoting factors that commit TAMs to a pro-tumoral phenotype, with production of IL-4, IL-13 (mainly produced by tumour-infiltrating Th2 lymphocytes (40)), CCL2 and IL-6, all of which further favour tumor survival and M2 polarization within the tumour environment

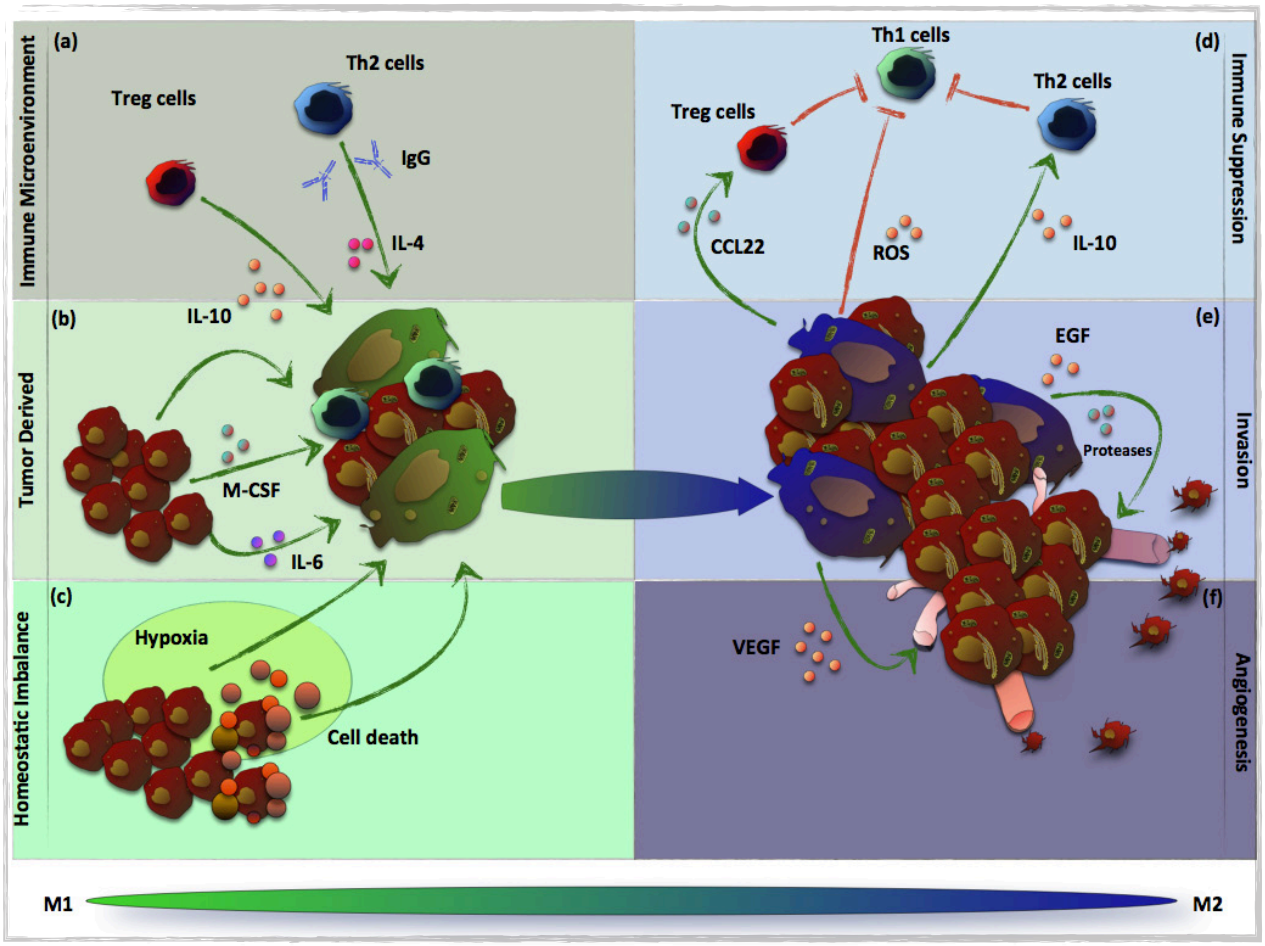


Figure 4. Factors that promote the polarization of TAMs towards a pro-tumor phenotype (a–c) can be subdivided into those derived from the immune system, actively produced by tumor cells, or resulting from tissue stress. These signals all direct the pro-tumor functions of TAMs (d–f) including immune suppression, tumor cell dissemination, and promoting angiogenesis. (Adapted from Ruffell et al. (51))

(47). In this scenario, TAMs block cancer-induced immune responses, eliminate or switch M1 macrophages, limit innate immune responses by impairing NK cells and T cells activation, and display defective production of inflammatory cytokines. Moreover, they produce high levels of IL-10, that drives Treg generation (45, 48-51). Therefore, macrophages are capable of destroying cancer cells and promoting anti-tumoral immune responses through their antigen-presentation capacity, but might also contribute to tumor progression via immune (37) and non-immune mechanisms (51) (promotion of angiogenesis (52), facilitation of tumor cell invasion and metastasis (53), protection of tumor cells from chemotherapy-induced apoptosis (54)) (fig.4). As a consequence, TAM polarization represents a critical process in tumor development, and has become the target for the development of anti-tumoral therapeutic strategies.

1.3 MACROPHAGE POLARIZATION MARKERS

A number of well-known markers are currently used to define the polarization state of mouse macrophages. Murine M1 "classically activated" (exposed to LPS and/or IFNγ) macrophages are usually identified by their high expression of NOS2, CD11c or CCR7 (55-57). On the other hand, the IL-4- dependent M2 macrophages polarization is usually defined through the expression of Arginase-1, macrophage mannose receptor (CD206), the chitinase-like Ym1 molecule, and Fizz-1 (found in inflammatory zone-1) (57). The opposite expression of NOS2 and Arginase-1 has become the paradigmatic best-established difference between classical and alternative mouse macrophages in terms of metabolism: while M1 macrophages use arginine to catabolize bactericidal nitric oxide through the stimulation of inducible nitric oxide synthase (NOS2), Arginase-1-expressing M2 macrophages produce the polyamine precursors urea and ornithine, needed for collagen synthesis and cell proliferation, respectively (58).

There are, however, other markers that distinguish M1 and M2 metabolism. Regarding lipid metabolism, M2 macrophages, but not M1 macrophages, show a significant up-regulation of fatty-acid uptake and fatty acid oxidation (58). This difference has been correlated with the opposite expression of COX-2 and COX-1, considered as M1 and M2 markers, respectively (59). In addition, iron metabolism is differentially regulated in M1 and M2 macrophages. M1 macrophages are set to an iron-retention phenotype defined as $CD163^{Low}$ (haemoglobin/haptoglobin receptor), $Ferritin^{High}$ (involved in iron storage) and $Ferroportin^{Low}$ (that mediates iron export), a profile that agrees with their bacteriostatic and tumoristatic activity. By contrast, M2 macrophages exhibit an opposite phenotype, as they are set to an iron-export mode that supports immune-regulation, matrix

remodelling and cell proliferation (60, 61).

In the case of the glucose metabolism, M1 macrophages (activated with LPS or IFNγ) exhibit high expression of 6-Phospho-fructo-2-kinase (PKF2) and elevated glycolysis, probably to overcome their high-energy requirements and to override potentially hypoxic microenvironments 13,15. Along this line, chronic activation of bone marrow derived-macrophages increases intracellular succinate, that stabilizes HIF-1α and synergizes with Toll-like receptors for promoting an increment of glycolysis (62). On the contrary, M2-macrophages (activated with IL-4) display a stronger oxidative-glucose metabolism and a potent beta-oxidation in lipid metabolism 1,11. The contribution of the intrinsic glucose metabolism to macrophage polarization has been illustrated by the involvement of CARKL, an enzyme of the pentose phosphate pathway, in promotion of M2-polarization (63). Therefore, glucose metabolism regulation is linked and determines macrophage polarization (62, 63).

The search for human macrophage polarization markers has revealed "difficulties of mouse-to-human extrapolation" (4). The comparison of human and mouse macrophages by in vitro studies demonstrated that only 26% of the polarization-associated genes are conserved between both species (56), claiming for the need of human macrophage polarization markers with potential diagnostic and therapeutic value. Numerous groups, including our own, have addressed the identification of such polarization markers in the last decade through transcriptional profiling of *ex vivo*-isolated macrophage populations and monocyte-derived macrophages generated *in vitro* under the influence of various M1 and M2-polarizing cytokines (IFNγ, GM-CSF, LPS, IL-4, M-CSF, IL-10, etc.) (3, 4, 37, 64). However, although the markers between human and mouse macrophages are not always comparable, the functions attributed to M1o M2 macrophages are usually common in both systems. A recent study by Hamilton´s group has reported the comparison of the gene expression profiles of human and murine M1 (GM-CSF) and M2 (M-CSF) macrophages generated from monocytes or bone marrow cells, respectively. Their study indicates that genes such as *CCL1, CCL5, CCL22, CCR6, CSF1, FLT1* and *ADORA1* are more highly expressed in human M1 macrophages, while *IL-10, THBS1, ALK, DLL1, IGF-1, ADRB2* and *MSR1* are preferentially expressed by M2 macrophages (56). Moreover, other groups (including ours) have reported that CD163, LXR, CD200R, CD36, c-Myc, FRβ, HO1 and DC-SIGN are preferentially expressed by human anti-inflammatory M2 macrophages as well as by tumour-associated macrophages (TAM) (2, 64-70), and that CD80, Activin A, PHD3, IRF5 and IRF4 are preferentially expressed in human M1 macrophages (56, 69, 71-73).

1.4 FACTORS AND SIGNALING PATHWAYS THAT UNDERLIE MACROPHAGE POLARIZATION

A network of signalling molecules, transcription factors, epigenetic mechanisms and post-transcriptional regulators underlies the existence of distinct M1 and M2 polarization/activation states (fig.5).

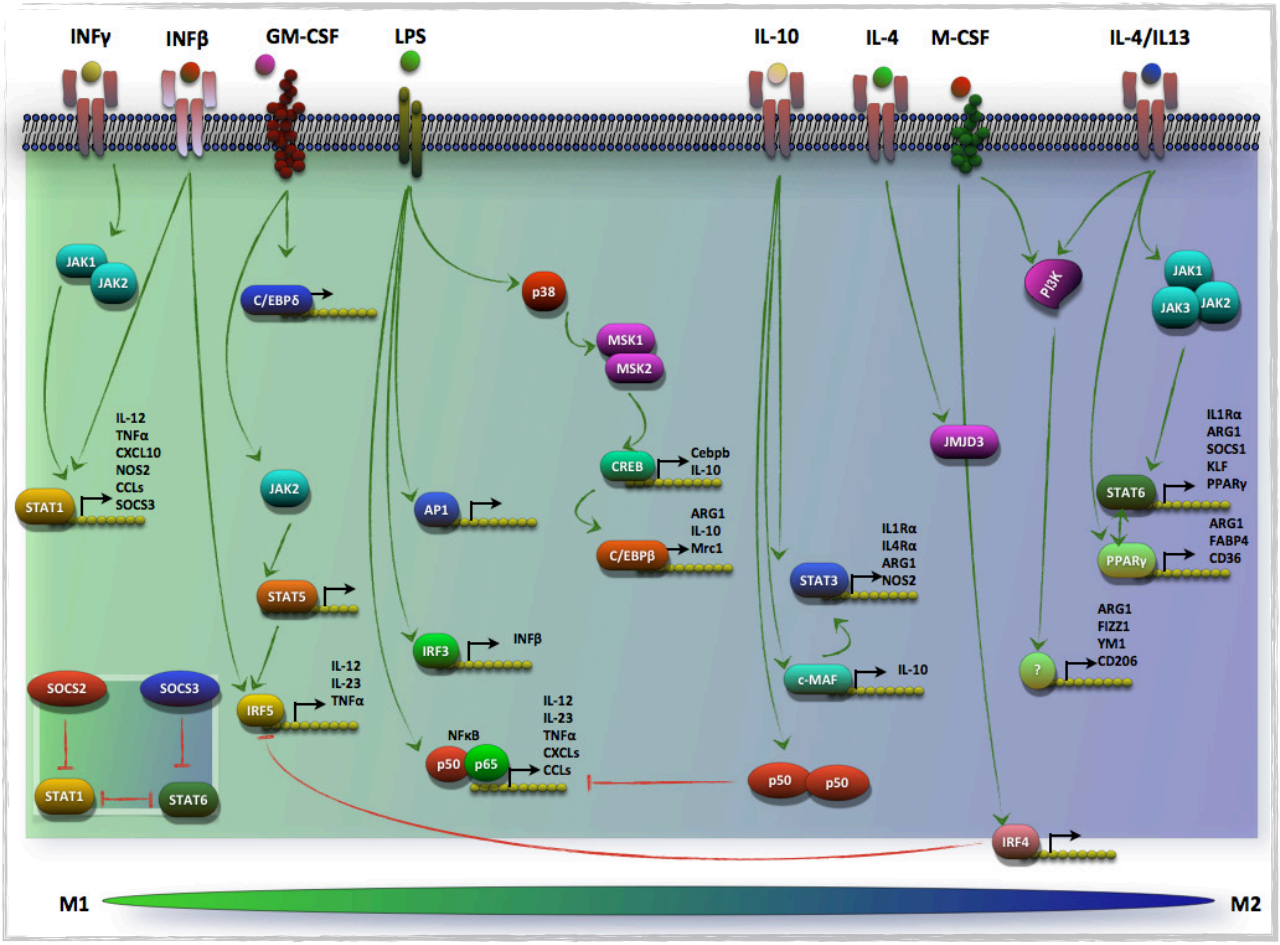


Figure 5. Signalling pathways that underlie macrophage polarization

- *NFκB signalling g pathway*

In M1 polarization, macrophages respond to TLR ligands (like LPS) by activating the NF-κB transcription factor family (RelA/p65, c-Rel, RelB, p50 and p52) (74). By contrast, the NF-κB signalling pathway seems to be absent or defective in M2 polarization, where NF-κB p50 homodimers are enhanced (75-77). NFκB p50 homodimers enhance IL-10 gene transcription, inhibit the NFκB-dependent production of IFNβ and negatively regulate STAT1 activity, whereas their absence leads to increased levels of TNFα and IL-12 (77, 78). Therefore, NFκB p50 homodimers impair M1 macrophage-mediated responses (75) and promote a defective expression of inflammatory NFκB-dependent cytokines and, consequently, an IL-10high/IL-12low M2-like profile (76, 77).

- *Interferon-related pathways: IRF and STAT signalling*

The TRIF/IRF3/INFβ/STAT1 axis is also involved in macrophage polarization (35, 79). Stimulation of the IFNγ receptor triggers a JAK-mediated tyrosine phosphorylation and the subsequent dimerization of STAT1, which promotes the expression of "classical" M1 genes such as NOS2, MHC class II transactivator (CIITA), IL-12 and TNFα, among others (80). Although TAMs are considered to display an M2-skewed phenotype and present low expression of M1-asociated genes, high levels of expression and activation of IRF3 and STAT1 has been found in some TAMs (76), suggesting that the IRF3/STAT1 axis might exert a dual role in macrophage polarization. Several studies have also suggested that the expression of IL-10, which characterizes M2 macrophages and is required to avoid damage caused by an excess of pro-inflammatory cytokines, can be promoted in some settings by the TRIF/IRF3/INFβ/STAT1 pathway, thus highlighting the critical role of STAT1 in both initiating and limiting M1 macrophage polarization (76, 81).

The contribution of IRF5 to human M1 polarization is somewhat controversial. Whereas Udalova´s group provided evidence for IRF5 as required for the acquisition of M1-associated genes and functions (73), others have found no evidence for such a role and propose that, instead, IRF4 might play that function (56). In fact, epigenetic studies have demonstrated the participation of the histone demethylase JMJD3 in the increased transcription of M2-associated genes triggered by IL-4, M-CSF or chitin, and that JMJD3 inhibits the IRF4-mediated transcription of typical M1-associated genes (82). Recently, it has been demonstrated that SOCS are also essential controllers of macrophage polarization, and that SOCS proteins regulate inflammatory responses. The use of SOCS2- and SOCS3-deficient macrophages have led to the finding that SOCS2 depletion causes STAT1 activation and enrichment in M1-like macrophages. Conversely, the lack of SOCS3 leads to enhanced STAT6 phosphorylation and accumulation of M2-like macrophages (83).

- *IL-4Rα/ JAK-STAT6/ PPAR pathway*

The role of IL-4 and IL-13-mediated signalling in M2 polarization is well established both in vivo and in vitro (84). IL-4Rα signals through a JAK-STAT6 pathway, and many of the M2-associated gene markers, such as Arg1 or Cd206, are regulated by this signalling route. Along the same line, the antagonism between STAT1 and STAT6 has been described for the Th1 and Th2 cell polarization induced by INFγ and IL-4, respectively (35, 80). STAT6 has been described to induce PPARδ in adipose tissue macrophages (ATM), driving them towards M2 polarization (85). Recent studies have shown that M2 macrophage polarization also appears to

be dependent on PPARγ and that Pparg-deficient macrophages display insulin resistance (86, 87). PPARγ is a master regulator of lipid metabolism in macrophages, as it inhibits pro-inflammatory gene expression through several mechanism, including trans-repression of NF-κB (80). At present, the collaboration of STAT6 and PPARγ for M2 macrophage polarization in murine macrophages is widely accepted (88).

- IL-10/ MAF and STAT3 pathway

Macrophage polarization in response to IL-10 is primarily dependent on c-MAF, STAT3 and the activation of NF-κB p50 homodimers. The expression of IL-10, a essential cytokine for resolution of inflammation, is regulated by PU-1, STAT3 and c-MAF 91,92.

- CREB and C/EBP

Other important factors involved in macrophage polarization are the CREB and C/EBP families of transcription factors. Both STAT6 and C/EBPβ are essential for Arg1 expression in macrophages in a stimulus-specific manner (Il-4/IL13 and TLR ligands, respectively). C/EBPβ also regulates the expression of M2-associated genes, since the CREB-dependent activation of the CEBPβ promoter is needed for the expression of M2 markers like Arginase-1, IL-10 (89-91) and SERPINB2 (92).

The identity of the signalling pathways implicated in the GM-CSF-promoted M1-polarization or the M-CSF-mediated M2-like polarization are still currently unclear 6,7,39. Recent studies have demonstrated the implication of C/EBPδ in the GM-CSF-prompted M1-polarization on bone marrow derived macrophages, where it inhibits M2 polarization (93). In the case of the M-CSF-induced polarization, it has been propossed that CREB might play a role in the process, since it contributes to IL-10 expression and because M-CSF-initiated signaling induces CREB phosphorylation (92).

- Signalling pathways activated in TAM

Like M2 macrophages, TAMs express high levels of M2-related genes such as CD163 (94-96). TAM polarization has been dissected at the transcriptional level in several models, including murine fibrosarcoma and human ovarian carcinoma (75). In those cases, TAMs display a defective M1 polarization caused by the nuclear accumulation of NF-κB p50 homodimers, which ultimately impair anti-tumoral responses and promote tumour growth (75, 76). On the other hand, murine fibrosarcoma-derived TAMs exhibit high levels of STAT1(76). In fact, STAT transcription factors are key players in the immunosuppressive phenotype of TAMs.

STAT6 appears to contribute to the pro-tumoral action of TAMs, since STAT6 KO macrophages display an M1 phenotype (97). STAT3 also importantly contributes to tumor survival and dissemination (98). In tumor-associated myeloid cells, STAT3 exhibits a pro-tumoral action by inducing IL-10, IL-23/p19 and inhibiting IL12/p35 expression (98, 99). Altogether, these observations point to STAT3 as a potential therapeutic target in cancer, as demonstrated in the case of human squamous cell carcinoma (100) and animal models of glioma (101) and melanoma (102).

With all of these signalling pathways acting together, the stimulation of an M2-to-M1 switch in TAMs would be a desirable strategy to improve anti-tumoral therapies. Thus, it has been described that TLR9-mediated activation of NFκB in combination with anti-IL-10 treatment promotes a switch in the polarization state of TAMs in a mouse mammary carcinoma model (103). Moreover, the fact that the M1-to-M2 polarization in TAMs might be driven by apoptotic-derived molecules (like S1P) within the tumour environment (104, 105) is in agreement with the reduced tumor progression detected after prevention of apoptotic cell recognition by macrophages (106). These and other pathways that might control the M1-to-M2 switch in TAMs are indicated in Figure 4.

An updated scheme illustrating all the factors whose involvement in macrophage polarization has been demonstrated or suggested is shown in Figure 6.

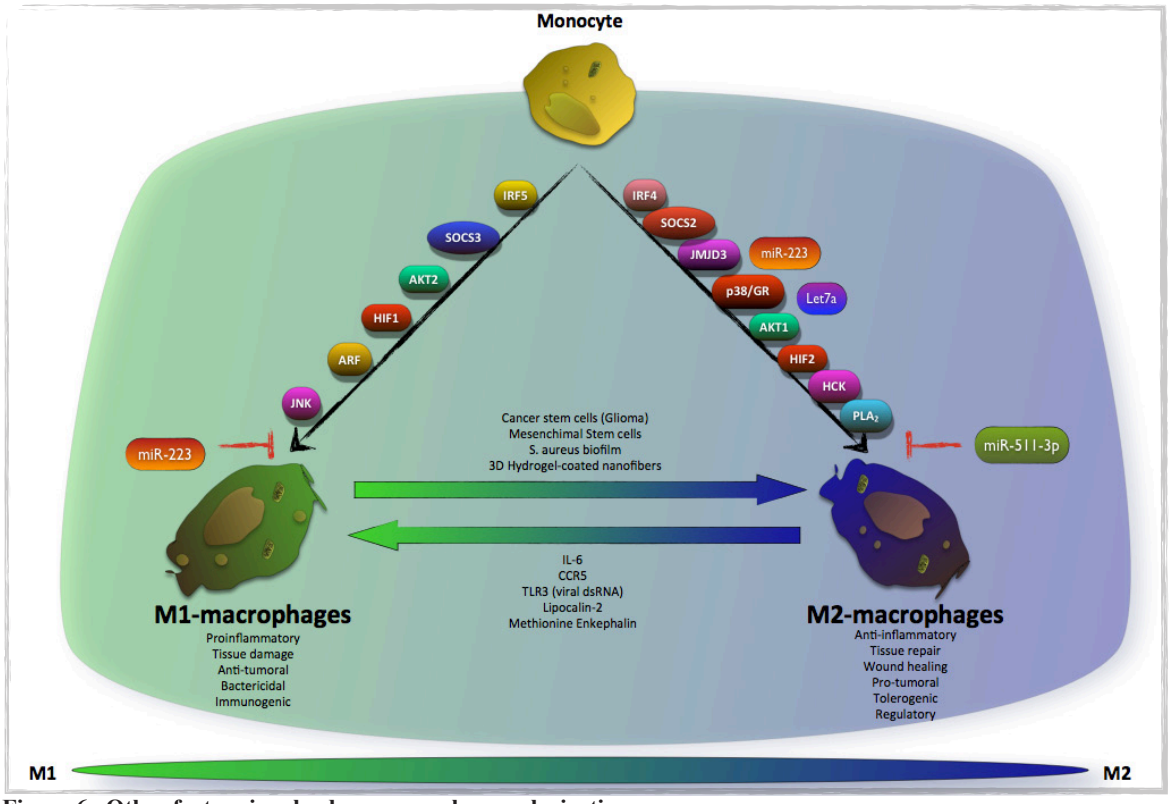


Figure 6. Other factors involved on macrophage polarization

2. SEROTONIN

The monoamine serotonin (5-hydroxytryptamine, 5-HT) has been known for more than a century (fig.7). However, although its role as neurotransmitter is well-established, evidences for its role in many fundamental aspects of physiology and behavior (mood, aggression, sleep, appetite, pain sensation, bone mass, tissue regeneration, platelet coagulation, gastrointestinal function and thermo-regulation) (107-110) are still being gathered. Approximately 2% of the tryptophan present in the diet is used for the synthesis of serotonin.

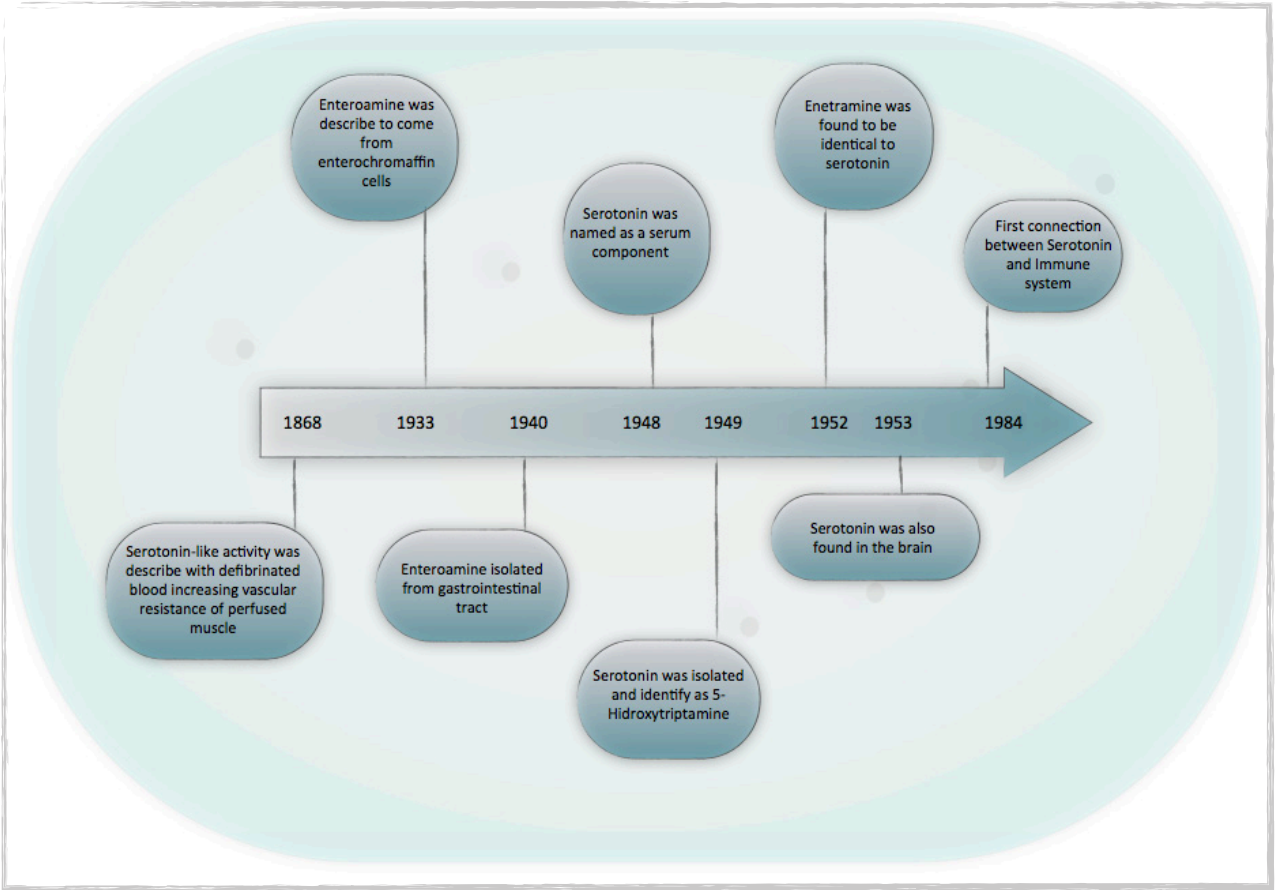
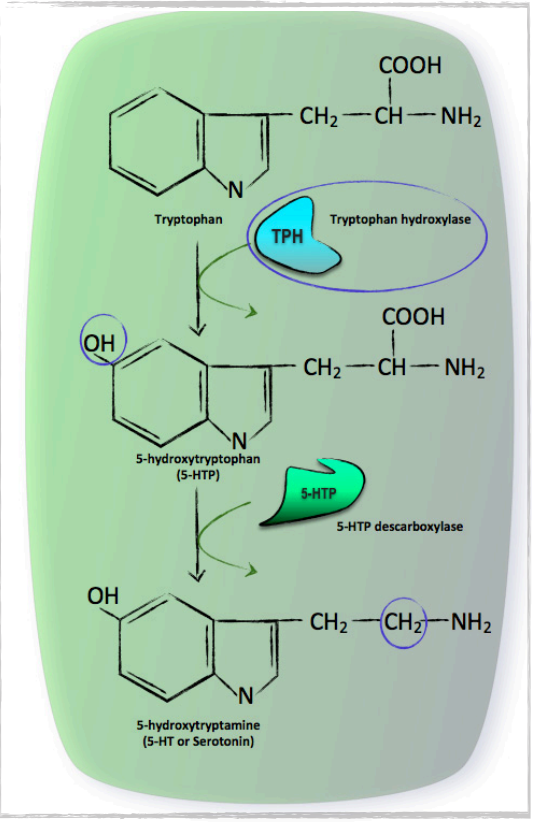


Figure 7. Timeline: advances on serotonin discovery and research.

The rate-limiting enzyme involved in the synthesis of 5-HT is tryptophan hydroxylase (TPH), which converts L-Tryptophan to 5-hydroxy-1-Tryptophan (fig. 8) through oxidation at position 5 of the pyrrole ring. A later decarboxylation mediated by 5-OH-tryptamine decarboxylase leads to 5-HT. Importantly, there are two isoforms of TPH: TPH-1, which is expressed in the pineal gland and peripheral tissues, and TPH-2, which is exclusively expressed in the dorsal raphe nucleus of the brain (109). TPH-1 provides 5-HT to non-neural cells whereas TPH-2 supplies 5-HT to the brain and mesenteric plexus, thus establishing the existence of two independent serotonin systems (brain and periphery), and whose independency is further supported by the hydrophobic nature of the molecule, that impairs crossing of the blood-brain barrier (111). However, it is still

currently unclear whether these two serotonin systems (brain, periphery) are completely independent, and some evidences suggest a certain level of communication between them.



In the brain, 5-HT is one of the most widely distributed neurotransmitters. Serotoninergic fibers originate in the brain raphe nuclei and their synaptic connections, where 5-HT mediates circadian rhythms and endocrine-related physiologic functions such as food intake, sleep, reproductive activity, cognition mood and anxiety (107). However, almost 95% of the whole body amounts of 5-HT is present outside the central nervous system, as it is produced by enterochromaffin cells (EC) of the gut (107). Once released from EC, 5-HT is taken up and primarily stored by platelets, but also by other cells like lymphocytes, monocytes, macrophages, mast cells and pulmonary neuroendocrine cells (109-111).

Figure 8. serotonin Synthesis

2.1 SEROTONIN RECEPTORS

Seven families of 5-HT receptors (5-HTR$_1$ to 5-HTR$_7$) have been described to mediate the physiological and pathological functions of 5HT. Up to 15 genes have been identified within these families, corresponding to a total of 20 subtypes with several alternative splicing variants (110). Differences in the tissue distribution of the 5-HTR subclasses appear associated with distinct activities and, probably, serve to fine-tune physiological and cellular responses to 5-HT. Thus, it has been suggested that each particular 5-HTR is probably linked to a specific biological response to 5-HT (110).

At the molecular level, 5-HT receptors are G protein–coupled receptors (GPCRs) (except for the 5-HTR$_3$ receptor, that is a ligand-gated ion channel). Therefore, 5-HT receptors possess seven transmembrane spanning helices, three intracellular and three extracellular loops, an extracellular amino-terminus, and an intracellular carboxy-terminal region (110). Functionally, the extracellular regions serve to bind the ligands. The intracellular

domains couple these receptors to various intracellular signaling effectors, with the various receptors triggering different intracellular signaling cascades and, as consequence, distinct functional outcomes (fig.9) (110). Since 5-HTR$_{2B}$ and 5-HTR$_{7}$ receptors are the subject of study of a portion of this work, their most relevant roles in physiology and pathology are outlined below.

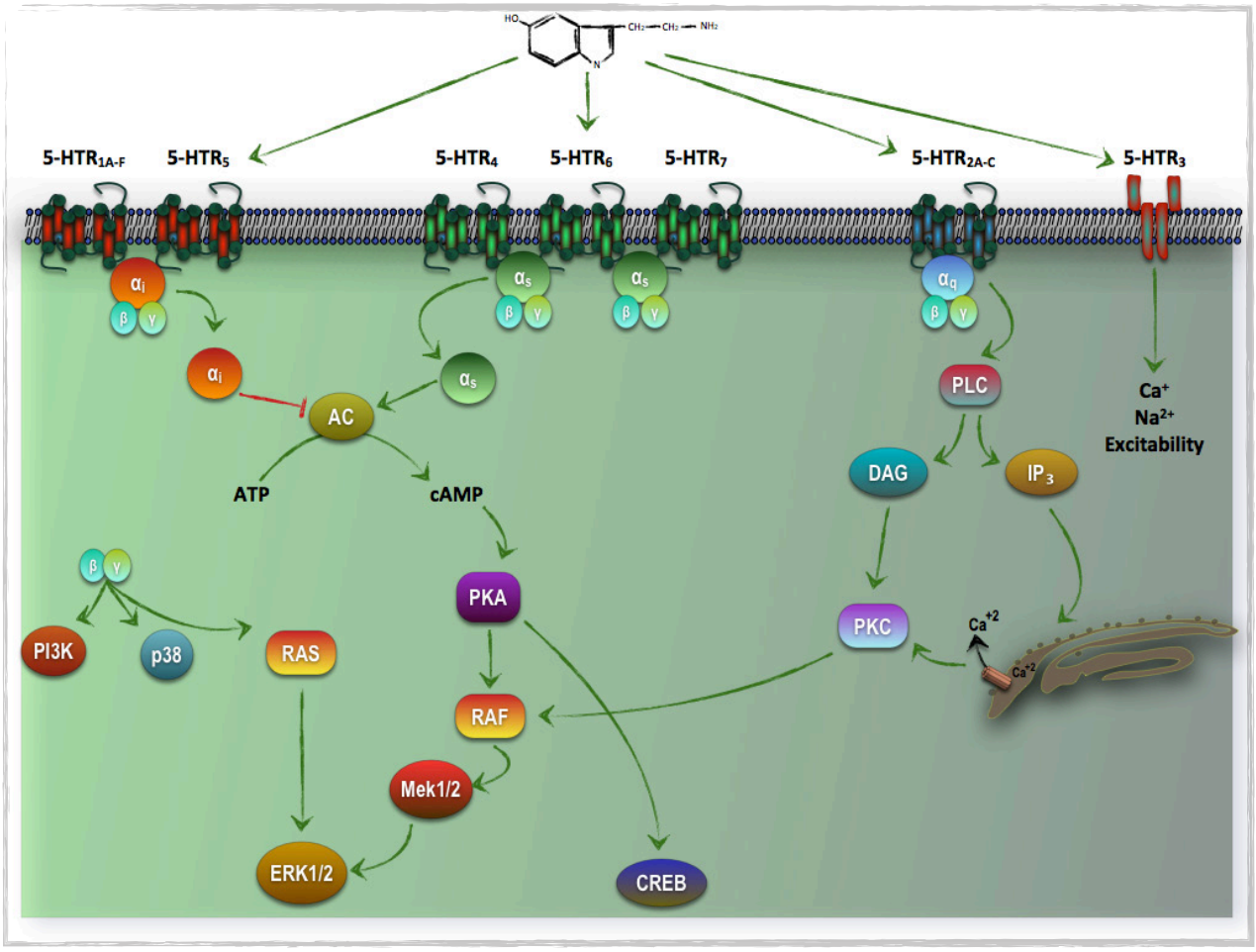


Figure 9. Molecular pathways that underlie serotonin receptors activation

2.2 5-HT$_{2B}$ RECEPTOR

5-HTR$_{2B}$ was first described as a 5-HTR$_{1}$-like receptor that mediated the 5HT-induced contraction of rat stomach fundus (112, 113). The human 5-HTR$_{2B}$ is homologous to 5-HTR$_{2A}$ and 5-HTR$_{2C}$ (45% and 42% identity, respectively). The 5-HTR$_{2B}$-encoding gene is located at chromosomal position 2q36.3–2q37.1 and includes two introns (114). Pharmacologically, the receptor binding properties of human 5-HTR$_{2B}$ compare well with those of the 5-HTR$_{2A}$ and 5-HTR$_{2C}$ receptors, although 5-HTR$_{2B}$ is clearly distinct (115). For example, 5-HTR$_{2B}$ has low affinity for ritanserin but higher affinity for yohimbine than 5-HTR$_{2A}$ or 5-HTR$_{2C}$. In the same line, SB 200646 and SB 206553 have high affinity for 5-HTR$_{2C/2B}$ and lower affinity for 5-HTR$_{2A}$, while spiperone

shows the opposite (116). Most importantly, a specific 5-HTR_{2B} antagonist (SB206741, 20-to-60-fold more selective for 5-HTR_{2B} than for other 5-HTR_2 receptors) and agonist (BW723C86, with about 10-times higher selectivity for the 5-HTR_{2B} versus 5-$HTR_{2A/2C}$) have now become available (116).

2.2.1 5-HTR_{2B} : EXPRESSION, SIGNALING AND PHYSIOLOGICAL ROLE

5-HTR_{2B} is strongly expressed during embryogenesis, mediating serotonin actions that are essential for normal development (117, 118). In adults, 5-HTR_{2B} is mainly distributed in peripheral organs, but its expression has been also detected in some restricted areas of the brain (only at the mRNA level) (110). The activation of 5-HTR_{2B} leads to the stimulation of phospholipase C (PLC) pathway via the alpha subunit of the Gq GTP-binding protein. 5-HTR_{2B} stimulates ERK 1/2 (p42/44) and, in 5-HTR_{2B}-expressing mouse fibroblasts, this activation is dependent on G protein and p21Ras(119). 5-HTR_{2B} also activates the oncogenic cytoplasmic tyrosine kinase p60Src, which induces both cyclin D1 and cyclin E via MAPKs-dependent or -independent pathways, respectively (120). These cyclins lead to the induction of retinoblastoma protein (pRB) and the transcription factor E2F, which subsequently activates the transcription of genes involved in DNA replication. The activation of the 5-HTR_{2B} downstream targets p21Ras and p60Rsc, together with the contribution of PDGFR and ErbB-2 growth factor receptors, seems to play a pivotal role in 5-HTR_{2B}-induced mitosis (121). 5-HTR_{2B} has also been proposed to display anti-apoptotic activity via MAPKs and PI3 Kinase (PI3K). Consequently, it is accepted that 5-HT_{2B} receptors mediate cardiac development during embryogenesis, promoting proliferation and differentiation of cardiomyoblasts (121). In adult heart, some groups have proposed that 5-HTR_{2B} is involved in the progression of myocardial hypertrophic remodelling (122), and activation of 5-HTR_{2B} results in increased proliferation of interstitial cells of Cajal in vivo (123). Small intestinal neuroendocrine tumors (NET) also seem to exhibit 5-HTR_{2B}-dependent mitosis (124). 5-HTR_{2B} expression increases in maternal pancreatic islets during pregnancy, while blocking of 5-HTR_{2B} signaling in pregnant mice limits beta cell expansion and causes glucose intolerance (125). Therefore, 5-HTR_{2B}-dependent mitosis and its physiological implications are widely studied in many different patho-physiological settings.

Interestingly, $HTR_{2B}^{-/-}$ mice present embryonic and neonatal lethality. Histological analysis of these embryos revealed increased apoptosis in the heart and decreased cell number in the ventricular *trabeculae*, as well as abnormal sarcomeric organization in the subepicardial layer (126, 127). Moreover, treatment with a 5-HT_2

receptor inverse-agonist in developing mouse embryos induced apoptosis in other tissues such as cephalic region, neural tube and heart (121). These $HTR_{2B}^{-/-}$ mice have been also shown to display reduced bone density, thus confirming the involvement of the receptor in osteogenesis (128). Altogether, these data have established a crucial role for 5-HT_{2B} in development.

Finally, the role of 5-HTR_{2B} in the nervous system has not been completely elucidated. Some studies implicated this receptor in the control of the respiratory network, because the local administration of a 5-HT_{2B} specific agonist in the pre-Bötzinger complex increased respiratory frequency (129, 130). However, $HTR_{2B}^{-/-}$ mice respiratory activity appeared to be unaltered, thus indicating that 5-HTR_{2B} is not the only serotonin receptor implicated in this process. In fact, 5-HTR_{2B} is present in all respiratory nuclei and found to be co-expressed with lower levels of 5-HTR_{2A} in many cells. Once the levels of circulating serotonin are higher than those required to activate 5-HTR_{2A}, 5-HTR_{2B} receptor might become activated to modulate the respiratory rhythm in a dose-dependent manner (130).

2.2.2 5-HTR_{2B} IN PATHOLOGY

In the 1980s and 1990s, the administration in the diet of the effective appetite suppressant fenfluramine was widely used until its use was linked to valvular heart disease (VHD) and Pulmonary arterial hypertension (PAH) (131, 132). Patients taking the drug combination fenfluramine/phentermine (Fen-Phen) for 1 to 28 months developed heart valve abnormalities, with high myofibroblast proliferation (131). Later studies reveled that fenfluramine and its metabolite norfenfluramine were potent agonists of 5-HTR_{2B}. Therefore, it was concluded that the activation of 5-HTR_{2B} on heart valve interstitial cells leads to the formation of proliferative foci and subsequent changes that compromise tissue functions (e.g. increased extracellular matrix deposition and leukocyte infiltration) (133, 134).

Pulmonary arterial hypertension (PAH) is a progressive and fatal disorder in humans that results from an increase in pulmonary blood pressure associated with abnormal vascular proliferation (135). Analysis of a chronic-hypoxic-mouse model demonstrated that hypoxia-dependent increase in pulmonary blood pressure and lung remodeling are associated with a serotonin- and 5-HTR_{2B}-dependent increase in vascular proliferation, elastase activity and TGFβ levels (136-138). More recently, bone marrow progenitor cells have been implicated

in the etiology of PAH (139). Thus, it seems that 5-HT contributes to the pathogenesis of PAH by activating bone marrow progenitor through 5-HTR_{2B} and ultimately leading to pulmonary vascular remodeling (140). Altogether, these observations point to 5-HTR_{2B} receptors as key mediators of serotonin-induced proliferation, which leads to pathology in certain tissues.

2.3 5-HTR_7 RECEPTOR

5-HTR_7 was identified as a serotonin receptor by several independent laboratories in 1993 (141-143). Since its discovery, 5-HTR_7 has been described in numerous species, including humans. 5-HTR_7 is highly expressed in the brain, particularly in the neocortex, hippocampus, and hypothalamus, as well as in the suprachiasmatic nucleus (144). In periphery, 5-HTR_7 has been detected predominantly in smooth muscle cells of the cardiovascular (138), gastrointestinal (145) and reproductive system (146, 147) , and in corneal epithelial cells (148). The 5-HTR_7 gene is located on human chromosome 10q21–q24 and contains three introns. Alternative splicing only occurs at the second and third intron and gives rise to at least five splice variants in human, mouse, and rat, which differ in their C-terminal tail. So far, three splice variants have been identified in human (5-HTR_{7A}, 5-HTR_{7B}, and 5-HTR_{7D}), three in mouse (5-HTR_{7A}, 5-HTR_{7B}, and 5-HTR_{7C}), and four in rat (5-HTR_{7A}, 5-HTR_{7B}, 5-HTR_{7C}, and 5-HTR_{7E}) (144). The three human splice variants encode proteins of 448 (5-HTR_{7A}), 435 (5-HTR_{7B}), and 479 (5-HTR_{7D}) amino acids. In general, the 5-HTR_{7A} isoform is more widely expressed, followed by the 5-HTR_{7B} variant, while the 5-HTR_{7C} and the 5-HTR_{7D} isoforms frequency is low. Splicing variants do not seem to possess functional differences and are pharmacologically indistinguishable (144). The availability of selective 5-HTR_7 ligands has been the limiting factor for elucidating the functions of this receptor. It is well established that 5-Carboxamidotryptamine (5-CT) and 8-OH-DPAT act as agonists for 5-HTR_7, but they can also activate other serotonin receptors. Fortunately, in the last years, 5-HTR_7-specific agonists have been described, including LP-12 (149) and AS-19 (150). On the other hand, selective antagonists of 5-HTR_7, such as SB-258719 and SB-269970, have also allowed the analysis of the activity and functions of this receptor (110, 144).

2.3.1 5-HTR$_7$: EXPRESSION, SIGNALING AND PHYSIOLOGICAL ROLE

As other serotonin receptors (5-HTR$_4$ and 5-HTR$_6$), 5-HTR$_7$ is a G protein–coupled receptor that interacts with Gα12. Its activation leads to stimulation of adenylyl cyclase, resulting in the conversion of ATP to cyclic AMP (cAMP) (144), an ubiquitous intracellular messenger that interacts with numerous targets, including the phosphorylating enzyme protein kinase A (PKA) (151) and the exchange proteins activated by cAMP (Epac) (152). PKA phosphorylates cAMP-responsive transcription factors, such as the cAMP response element binding protein (CREB), thus affecting gene expression, whereas Epac activates Rap and Ras GTPases (110). It has been demonstrated that the activation of the 5-HTR$_7$ Gα12 signalling pathway also leads to stimulation of Cdc42 and RhoA, resulting in serum response element-mediated gene transcription, which in turn induced filopodia formation and cell rounding (153). Ligation of 5-HTR$_7$ also activates ERK1/2 but, in this case, the pathway differs depending on the cell system (151, 152). 5-HT induces a rapid 5-HT$_7$–dependent phosphorylation of ERK and IκBα that result in increased early T-cell activation and proliferation (154). In the case of astrocytoma and microglial cell lines, the stimulation of 5-HT$_7$ results in the expression of IL-6 via p38 and PKC activation (155, 156).

The activation of 5-HTR$_7$ in the central nervous system and in the periphery modifies different cellular functions. In periphery, 5-HTR$_7$ activation mediates smooth muscle relaxation of the human colon (110, 157). A recent study has shown 5-HTR$_7$ expression in hepatocytes, where it promotes IGF-1 synthesis via cAMP/CREB/AKT (158) in response to elevated concentrations of 5-HT. In collaboration with the central nervous system, peripheral 5-HTR$_7$ was also found to play a role in the regulation of the micturition reflex (159, 160), and also influences the resolution of gut inflammation, since the blockade of 5-HTR$_7$ in dendritic cells improves the resolution of inflammation (161). In the central nervous system, studies with HTR$_7$ $^{-/-}$ mice and selective antagonists (SB-269970) demonstrated that 5-HTR$_7$ is involved in a number of functions. In depression conditions, HTR$_7$ $^{-/-}$ mice exhibit a “antidepressant-like” phenotype (162), and in circadian rhythms and sleep, 5-HTR$_7$ antagonists increase the time to onset of REM sleep and reduce the time spent in REM (163, 164). Antagonists of 5-HTR$_7$ block 5-HT-induced hypothermia in both guinea pigs and rats. The role of 5-HTR$_7$ in thermoregulation has been confirmed in HTR$_7$ $^{-/-}$ mice, where 5-HT or 5-HTR$_7$ agonists fail to produce hypothermia (165). Moreover, 5-HTR$_7$ has been involved in anxiety, schizophrenia, nociception, epilepsy, and memory (144).

2.4 SEROTONIN AND IMMUNE SYSTEM

The determination of the role of 5-HT outside the central nervous system (CNS), and especially in immune cells, has been one of the recent aims when analyzing the neuro-immune connection. As mentioned above, 90% of whole body serotonin is produced by enterochromaffin cells, and about 98% of the remaining 5-HT is found in platelets, whereas only 2% is located within CNS (108). In platelets, 5-HT is stored and constitutes a major secreted product. Under physiological conditions, the plasmatic and vascular concentrations of 5-HT are maintained at low levels by mechanisms like uptake, storage or monoamine oxidases-mediated degradation (107). Under inflammatory conditions like thrombosis and ischemia, activated platelets release 5-HT and increase its concentration around the inflamed area (108, 166). Moreover, pro-inflammatory stimuli (LPS, IFNγ) directly induce platelet activation, which further enhances 5-HT levels (167, 168).

2.4.1 CELLULAR AND FUNCTIONAL EFFECTS OF SEROTONIN IN IMMUNE CELLS

By acting through different receptors, 5-HT has an immunomodulatory role because of its stimulatory or inhibitory activities on B and T lymphocytes, NK cells and monocyte/macrophages/dendritic cells (fig.10). 5-HT induces adhesion and chemotaxis in mouse and human mast cells, promoting their migration towards inflammatory sites through 5-HTR_{1A} (169). The infiltration and migration of eosinophils also seems to be 5-HTR_{2A}-dependent (170, 171), as the lack of 5-HTR_{2A} diminishes allergen-induced pulmonary eosinophilia in allergic asthma (171). Furthermore, 5-HT enhances the cytolitic function of NKs in vitro (172), and long-term treatment with serotonin reuptake inhibitors increases NK cell proliferation (173). It has also been shown that 5-HT is shuttled from dendritic cells to T lymphocytes as a means to modulate activation, proliferation, and differentiation through 5-HTR_7 (154), and that 5-HT might be necessary for optimal macrophage accessory function (174).

5-HT, acting through 5-HTR_1 and 5-HTR_2 receptor families, induces chemotaxis in immature human DCs and enhances the migration of pulmonary DCs to draining lymph nodes in mice (175). In the case of macrophages, physiological concentrations of 5-HT suppress IFNγ–induced MHC class II expression and phagocytosis in murine macrophages (176-178). In the context of inflammatory pathologies, 5-HT regulates macrophage-mediated angiogenesis by reducing MMP12 expression in tumor-infiltrating macrophages (179). 5-HT can also

modulate the production of chemotactic factors and cytokines from various immune cells (180). Therefore, 5-HT participates in the control of numerous events during inflammatory processes.

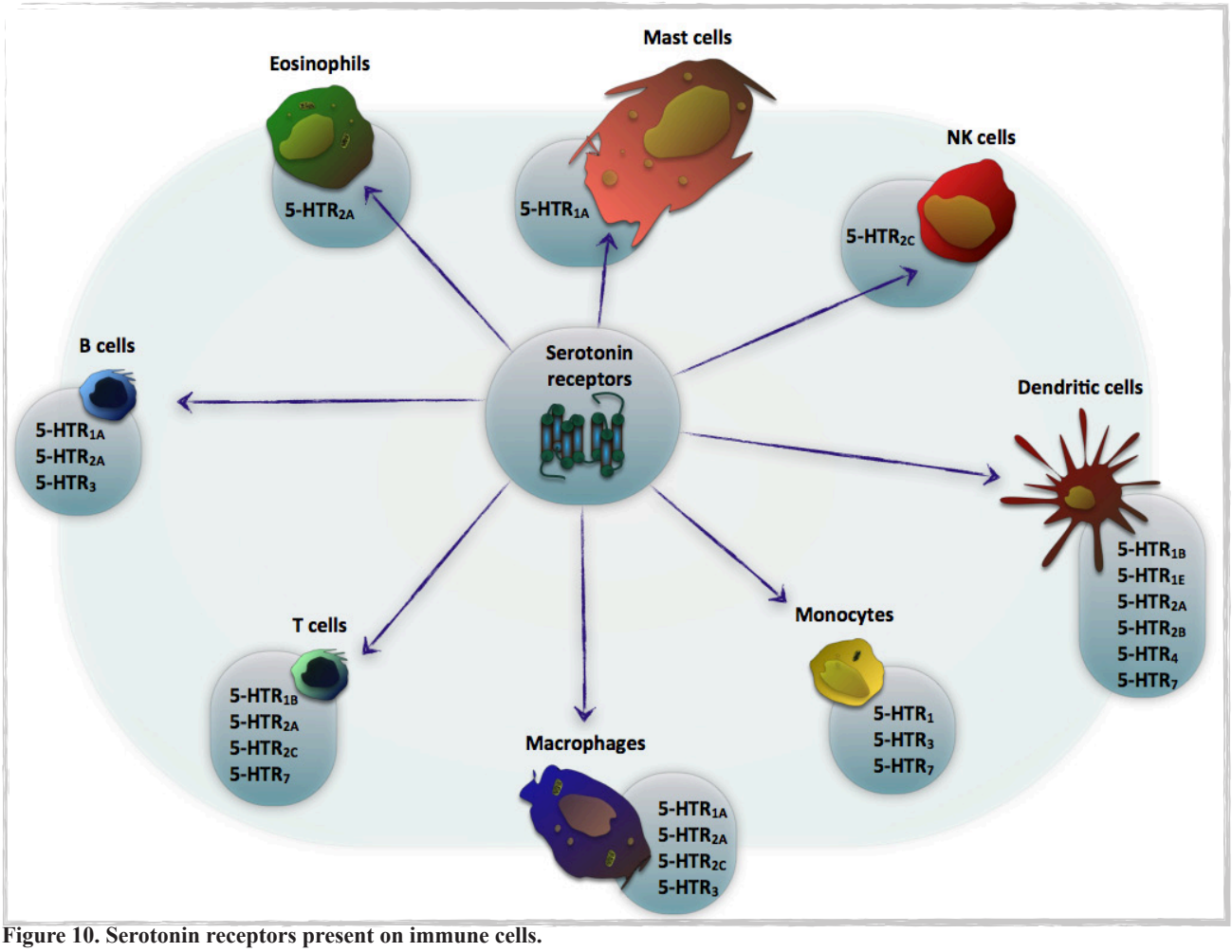


Figure 10. Serotonin receptors present on immune cells.

2.4.2 5-HT AND CYTOKINE RELEASE

The balance between pro-inflammatory and anti-inflammatory cytokines is crucial in the control of inflammatory responses. As described above, the presence of 5-HT at inflammatory sites suggests its possible involvement in the control of the inflammation promotion/resolution equilibrium. The effect of 5-HT on immune cells has been studied in different cell models and most in vitro results have been obtained in the presence of fetal bovine serum. A potential experimental problem with previously published information, only recently appreciated, is the presence of considerable amounts of 5-HT in culture media, primarily derived from serum or from 5-HT producing cells. Indeed, 10% of heat-inactivated foetal bovine serum contains 300 nM 5-HT as detected by ELISA (109). These levels of 5-HT, assuming that immunoreactive 5-HT is bioactive, are sufficient to activate many 5-HT receptors. Thus, there is real danger that contaminating 5-HT may have altered experimental results published in the past.

In the case of NK cells, 5-HT increases the production of IFNγ in the presence of monocytes through the activation of 5-HTR_{1A} (181), but suppresses the production of this cytokine in whole blood cells (182). In whole blood, 5-HT decreases TNF-α and IL-6 production and has no effect on the LPS-induced production of IL-10 (183, 184). Along this line, human CD14+ monocytes respond to 5-HT by increasing the production of LPS-stimulated IL-1β and IL-8, and by decreasing that of TNF-α (185).

Several studies have also revealed a role for 5-HT in DC cytokine secretion. Thus, 5-HT alters the cytokine profile of DCs, enhancing IL-1β, IL-8, IL-6 and IL-10, and decreasing IL-12 and TNF-α (175, 186). It has also been demonstrated that 5-HT impairs GM-CSF/IL-4–driven human monocyte-derived dendritic cell (MDDC) differentiation by reducing co-stimulatory molecule and CD1a expression and Mixed Lymphocyte Response stimulatory activity, and by increasing CD14 levels and IL-10 production through 5-HTR_1 or 5-HTR_7 (187). In addition, 5-HT-treated DCs increase their production of the Th2 attracting chemokine CCL22 while decreasing that of the Th1 chemokine CXCL10 (175). Thus, DCs treated with 5-HT induce Th2 polarization in naïve CD4 T cells (175). However, recent studies have shown that gut DC produce IL-12 in response to 5-HT and LPS (188), and that the lack of 5-HTR_7 ameliorates mucosal inflammation (161).

Regarding macrophages, 5-HT modulates many of their effector functions, including enhancement of phagocytosis (189). 5-HT decreases the LPS-evoked production of TNF-α and IL-6 in murine peritoneal macrophages (190) and, in 5-HTR_{2C}–expressing alveolar macrophages, upregulates the expression of CCL2 (191). In human alveolar macrophages and macrophage-like synovial cells, 5-HT stimulation leads to overexpression of PGE2 (192, 193), enhances LPS-stimulated IL-10 production and decreases LPS-induced TNF-α secretion (193). Therefore, and as a whole, 5HT exhibits a plethora of effectors functions on immune cells, and especially on cells within the myeloid lineage. The effects that 5-HT receptor ligation exerts on human macrophage polarization constitutes a major topic of the present thesis.

3. INTRAVENOUS IMMUNOGLOBOLINS (IVIG)

Intravenous immunoglobulin (IVIg) is a polyclonal IgG fraction pooled from the sera of thousands of healthy donors. Both poly-reactive natural antibodies and antibodies with specificities for allotypic antigens are present in the pool. IVIg is used as a replacement therapy in immunodeficient individuals, who are unable to mount their own effective immune responses. Less intuitively, IVIg can also be used to suppress the pathological immune responses that occur in patients with autoimmunity, thus exerting a potent immunomodulatory action. IVIg therapy is FDA-approved for a limited number of pathologies and it is beneficial in other several pathologies (194, 195) (Table 1). Most experimental systems have provided evidences that the active immunomodulatory component of IVIg resides within the Fc domain-containing fractions rather than the F(ab´)2 fractions, thus implicating Fc receptors in the therapeutic benefits of IVIg. Although monomeric IgG is the major constituent, IVIg contains small percentages of dimeric and polymeric IgG, and even immunocomplexes (196). Moreover, it has been also shown that a small fraction of IgG in IVIg contains Fc-bound sialic acid and that this small fraction plays a critical roll in IVIg-mediated responses (194, 197-199). So far, all these reactive parts have been claimed to contribute to the IVIg immune-modulatory ability (200, 201). Importantly, previous studies have shown that IVIg impairs the metastatic spread of various carcinomas in mice, and contributes to tumor regression in a number of cancer patients, leading to the proposal of IVIg as a potential anti-metastatic drug (202, 203).

FDA-approved indications	Additional approved indications with criteria	
Primary immunodeficiency dissease	Neuromuscular disorders	*Refractory dermatomyositis*
Chronic lymphocytic leukemia	*Guillain-Barre syndrome*	Hematologic disorders
Pedriatic HIV infection	*Relapsing-remitting multiple sclerosis*	*Autoimmune hemolytic anemia*
Kawasaki´s disease	*Myastemia gravis*	*Severe anemia associated with parvovirus B19*
Allogeneic bone marrow transplantation	*Refractory polymyositis*	*Autoimmune neutropenia*
Chronic inflammatory demyelinating polyneuropathy	*Polyradiculoneuropathy*	*Neonatal alloimmune thrombocytopenia*
Kideney transplantation involving a recipient with a high antibody titer or an ABO-incompatible donor	*Lambert-Eaton myasthemic syndrome*	*HIV-associated thrombocytopenia*
Multifocal motor neuropathy	*Opsoclonus-myoclonus*	*Graft-versus-host disease*
	Birdshot retinopathy	

Table 1. Pathologies for wich IVIg is FDA-approved

3.1 IVIG : MECHANISM OF ACTION

The mechanisms of action of IVIg are still poorly defined. Even more, many of the diseases that respond to IVIg show pathologic profiles that differ from one another. These circumstances have precluded so far the identification of a single common IVIg mechanistic pathway that might apply to every clinical scenario. Consequently, many distinct and not-mutually exclusive mechanisms of action of IVIg have been proposed (204). In fact, it is possible that the benefits of IVIg cannot be explained by a unique and common mechanism. Indeed, pleiotropic effects of IVIg might be responsible for the success of IVIg therapy to treat many different inflammatory and autoimmune diseases (204). As an example, administered IVIg has been shown to exert both anti-inflammatory and pro-inflammatory actions (204). In general, anti-inflammatory activities have been observed when IVIg is administered at high doses, whereas pro-inflammatory effects can be seen with low IVIg doses. This last effect involves complement activation and FcγR (204, 205). What follows is a concise compendium of the mechanisms of action of IVIg described so far, emphasizing only those with a more obvious relationship to the present work.

Fab-mediated activities (Fab: antigen-binding fragment of IgG) of IVIg

Since IVIg includes many antibodies from different donors and with very distinct specificities, one possible explanation of its therapeutic benefits may rely on the fact that the Fab portion binds to a wide spectrum of proteins or cell-surface receptors. In this regard, IVIg has been shown to exert several Fab-mediated activities (fig.11):

1. *Suppression or neutralization of autoantibodies and cytokines* (206).

2. *Neutralization of activated complement components* (206). IgG binds to potentially damaging complement fragments and blocks deposition of these fragments on target tissues. Thus, IVIg can prevent immune damage that arises from cell destruction or inflammation.

3. Restoration of idiotypic/anti-idiotypic networks (207). IVIg contains an array of anti-idiotypic antibodies that can target B lymphocytes expressing these idiotypes, thus downregulating or eliminating autoreactive clones.

4. *Blockade of leukocyte adhesion molecules* (204).

5. *Targeting of specific immune cell-surface receptors* (204).

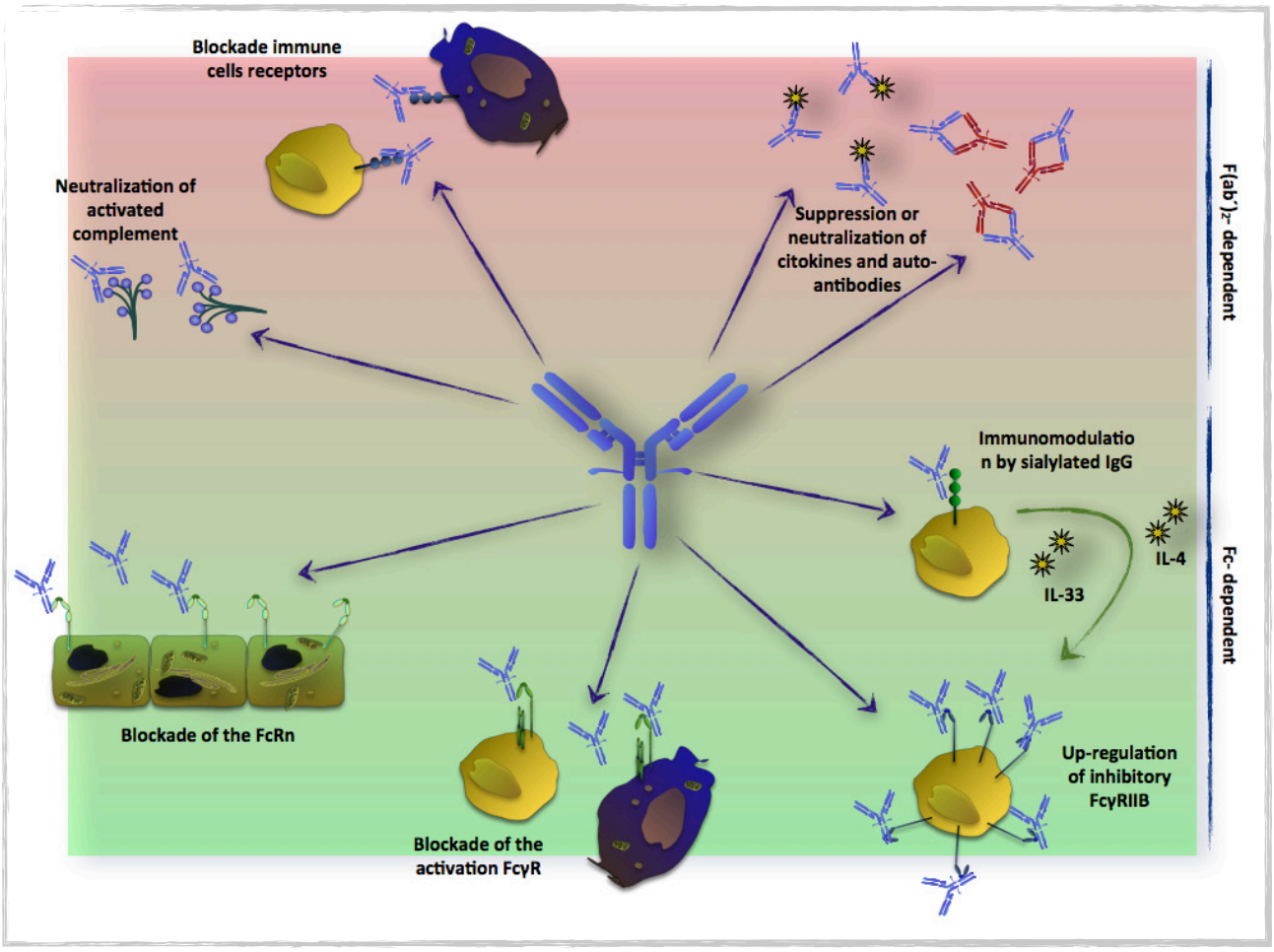


Figure 11. IVIg mechanisms of action.

Fc-dependent activities (Fc: crystallizable fragment of IgG) of IVIg

1. Blockade of the FcRn (neonatal Fc receptor). FcRn localizes in many tissues and is highly expressed on vascular endothelial cells. This receptor is critical to modulate IgG levels, since it attenuates the catabolism of IgG by preventing lysosomal degradation and allowing intact IgG return to the circulation(208). Indeed, it is possible that IVIg intercepts the interaction of autoantibodies to this receptor, so eliminating them from circulation more rapidly and reducing target-cell damage (204).

2. *Blockade of activating FcγR (receptor for the Fc portion of IgG)*. This explanation is one of the most obvious, since FcγR receptors are involved in many antibody-directed functions and its blockade can directly affect the pathology associated to immune system deregulation (197). Nevertheless, it is important to consider that FcγR in humans tend to be low-affinity receptors, so they would present a low ability to interact with monomeric IgG

(the main component of IVIg). In fact, IVIg preparations that contain dimeric or multimeric IgG could be more anti-inflammatory (196). However, the regulation of these receptors by IVIg can be indirect. IgG antibodies in IVIg preparations interact with their respective antigens creating a big pool of immune-complexes. These complexes might compete with autoantibody-antigen complexes, blocking their access to FcγR receptors and, consequently, modulating autoimmune responses (200).

3. *Modulation of maturation and function of dendritic cells*. It has been demonstrate DCs primed ex vivo with IVIG and then trans¬ferred into mice were able to ameliorate (immunothrombocytopenia) ITP to a similar extent to IVIG injection (209). Interestingly, IVIG-primed FcγRIII-deficient DCs were not able to suppress ITP (209). However, in other studies, in which FcγRIII-deficient mice were used in models of nephro¬toxic nephritis and ITP, IVIG activity was not impaired, indicating that additional pathways may be operative in vivo (210, 211).

4. *Up-regulation of inhibitory FcγRIIB*. Different experimental systems have demonstrated that IVIg increases expression of the inhibitory receptor FcγRIIB on effector macrophages. As it happens with the low-affinitty activating FcγR, a direct interaction between IVIg and FcγRIIB (also a low-affinitty receptor) is unlikely. However, the fact that IVIg modulates the activity of macrophages through the up-regulation of inhibitory FcγRIIB can explain part of the blockade of pro-inflammatory responses by IVIg (198, 212, 213).

5. *Immunomodulation by sialylated IgG*. One of the remaining questions to explain its anti-inflammatory action is why high doses of IVIg are required to promote anti-inflammatory actions. The observation that different patterns of IgG glycosylation can be found in animal models of inflammation and autoimmune disease patients shed light on a potential IVIg mechanism that would be dependent on unique and rare IgG glycoforms (214). Indeed, IVIg contains a small portion of sialylated Fc-containing IgG that was found to mediate the IVIg anti-inflammatory action in a mouse model of arthritis (215). In fact, deglycosylated IVIg was unable to exert an anti-inflammatory action (198, 216). This hypothesis is likely to support the presence of a novel receptor on regulatory macrophages that would specifically recognize sialic acid-rich IgG and promote an anti-inflammatory environment (199, 206, 217). To exert its anti-inflammatory action, sialylated Fc fragments appeared to require a specific lectin expressed on macrophages, SIGN-R1 in mice or DC-SIGN (dendritic-cell-specific ICAM 3-grabbing non-integrin) in humans (218). This lectin-dependent anti-inflammatory activity

of IVIg has been described to depend on two different macrophage populations: IVIg would first bind DC-SIGN or SIGN-R1 in an M-CSF-dependent macrophage subset, which subsequently secretes IL-33 to activate basophiles. These basophiles would then increase the levels of IL-4, leading to the activation of effectors macrophages that would up-regulate FcγRIIB and inhibit inflammation as described above (217). In spite of all these evidences, recent studies have suggest that the proposed molecular interaction between terminal Fc sialic acid and DC-SIGN is not the primary recognition event responsible for triggering the IVIg anti-inflammatory effect (219). Therefore, it seems that the contribution of the sialylated motifs of IVIg and their mechanism of action should be re-evaluated (219).

In the present work we have addressed the potential role of IVIg on human and murine macrophage polarization, and its effects on a pathological setting where macrophage polarization plays a relevant role (tumor growth

AIMS OF THE THESIS

and metastasis).

Aims of the Thesis

The principal aim of this Thesis was to analyze whether serotonin (5-HT) and Intravenous Immunoglobulin (IVIg) modify macrophage polarization at the phenotypic and functional level. For this purpose, the following objectives were addressed:

1. Identification of 5-HT receptors expressed by human monocyte-derived macrophages at different polarization states.
2. Analysis of the transcriptomic and functional effects of 5-HT on human macrophages.
3. Determination of the phenotypic and functional effects of IVIg on pro- and anti-inflammatory macrophages, both *in vitro* and *in vivo*.

Objetivos

El principal objetivo de esta Tesis Doctoral fue analizar cómo la serotonina (5-HT) y las Inmunoglobulinas Intravenosas (IVIg) son capaces de modificar la polarización de macrófagos a nivel fenotípico y funcional. Para esto, se abordaron los siguientes objetivos:

1. Identificación de los receptores de serotonina presentes en macrófagos derivados de monocitos humanos.
2. Análisis de los efectos de la serotonina sobre macrófagos a nivel trascriptómico y funcional.
3. Determinación del efecto de IVIg, tanto *in vitro* como *in vivo*, de los cambios fenotípicos y funcionales que provoca en macrófagos pro- y anti-inflamatorios.

RESULTS

The following results are presented in paper format, in wich the next scientific articles are included:

1. Serotonin skews human macrophage polarization through HTR2B and HTR7. J ***Immunol. 2013 Mar 1;190(5):2301-10. doi: 10.4049/jimmunol.1201133. Epub 2013 Jan 25.***
2. Serotonin-dependent gene expression profile in human macrophages. ***Forthcoming***.
3. Intravenous immunoglobulin promotes anti-tumor responses by modulating macrophage polarization. ***Under review on J. Immunol***

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Serotonin Skews Human Macrophage Polarization through HTR $_{2B}$ and HTR$_{7}$

Mateo de las Casas-Engel, Angeles Domínguez-Soto, Elena Sierra-Filardi, Rafael Bragado, Concha Nieto, Amaya Puig-Kroger, Rafael Samaniego, Mabel Loza, María Teresa Corcuera, Fernando Gómez-Aguado, Matilde Bustos, Paloma Sánchez-Mateos and Angel L. Corbí

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Serotonin Skews Human Macrophage Polarization through HTR_{2B} and HTR_7

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Besides its role as a neurotransmitter, serotonin (5-hydroxytryptamine, 5HT) regulates inflammation and tissue repair via a set of receptors ($5HT_{1–7}$) whose pattern of expression varies among cell lineages. Considering the importance of macrophage polarization plasticity for inflammatory responses and tissue repair, we evaluated whether 5HT modulates human macrophage polarization. 5HT inhibited the LPS-induced release of proinflammatory cytokines without affecting IL-10 production, upregulated the expression of M2 polarization–associated genes (*SERPINB2*, *THBS1*, *STAB1*, *COL23A1*), and reduced the expression of M1-associated genes (*INHBA*, *CCR2*, *MMP12*, *SERPINE1*, *CD1B*, *ALDH1A2*). Whereas only $5HT_7$ mediated the inhibitory action of 5HT on the release of proinflammatory cytokines, both $5HT_{2B}$ and $5HT_7$ receptors mediated the pro-M2 skewing effect of 5HT. In fact, blockade of both receptors during in vitro monocyte-to-macrophage differentiation preferentially modulated the acquisition of M2 polarization markers. $5HT_{2B}$ was found to be preferentially expressed by anti-inflammatory M2(M-CSF) macrophages and was detected in vivo in liver Kupffer cells and in tumor-associated macrophages. Therefore, 5HT modulates macrophage polarization and contributes to the maintenance of an anti-inflammatory state via $5HT_{2B}$ and $5HT_7$, whose identification as functionally relevant markers for anti-inflammatory/homeostatic human M2 macrophages suggests their potential therapeutic value in inflammatory pathologies. *The Journal of Immunology*, **2013, 190: 2301–2310.**

Although GM-CSF and M-CSF contribute to both cell survival and proliferation, they exert distinct actions during macrophage development. The lack of M-CSF alters the development of various macrophage populations (1), whereas GM-CSF–deficient mice only exhibit altered maturation of alveolar macrophages (2). Along the same line, both cytokines promote the in vitro differentiation of macrophages with distinct morphology, pathogen susceptibility, and inflammatory function (3–5). GM-CSF drives the generation of monocyte-derived macrophages that produce proinflammatory cytokines in response to LPS and display high Ag-presenting and tumoricidal capacity (M1-polarized macrophages). Alternatively, M-CSF yields macrophages that release IL-10 in response to pathogens and exhibit high phagocytic and protumoral activity (M2-polarized macrophages) (3, 6). Accordingly, and based on their respective cytokine and gene expression profiles, human macrophages generated in the presence of GM-CSF or M-CSF are considered as proinflammatory and anti-inflammatory, respectively (3, 7, 8), and the acquisition of their gene expression profiles is critically dependent on activin A and the ALK/Smad signaling axis (3, 7, 8).

Serotonin (5-hydroxytryptamine, 5HT) is synthesized from L-tryptophan via a rate-limiting reaction catalyzed by two tryptophan hydroxylases encoded by genes with a distinct pattern of expression (*TPH1* in periphery, *TPH2* in brain) (9). Outside the CNS, 5HT is synthesized and released into the circulation by enterochromaffin cells and is rapidly taken up and stored by platelets and, to a lesser extent, lymphocytes, monocytes, and macrophages (9). Serotonin signals through seven types of receptors ($5HT_{1–7}$), six of which belong to the G protein–coupled superfamily of receptors ($5HT_1$, $5HT_2$, $5HT_4$, $5HT_5$, $5HT_6$, $5HT_7$) and include ERK1/2 activation as a downstream signal (9, 10). As a neurotransmitter, 5HT regulates mood and behavior as well as many cardiovascular and gastrointestinal functions (9). However, 5HT is also a growth factor, and it exhibits mitogenic properties on smooth muscle cells (11), hepatocytes (12), and endothelial cells (13), mainly through activation of $5HT_2$ receptors.

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The online version of this article contains supplemental material.

Abbreviations used in this article: 5HT, 5-hydroxytryptamine; MDDC, monocyte-derived dendritic cell; MMP12, matrix metalloproteinase 12; LSEC, liver sinusoidal endothelial cell; PAH, pulmonary arterial hypertension; qRT-PCR, quantitative real-time PCR; TAM, tumor-associated macrophage.

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Additionally, 5HT functions as a regulator of inflammation and tissue regeneration and repair (14), and it modulates cytokine production in a cell type–dependent manner. In peripheral blood, serotonin is released from platelets and lymphocytes/monocytes following stimulation by LPS and IFN-γ (15), and it modulates cytokine production by myeloid cells (16). Physiologic concentrations of 5HT suppress IFN-γ–induced MHC class II expression and phagocytosis in murine macrophages (17, 18) and inhibit the LPS-induced IL-1β, IL-6, IL-8, IL-12p40, and TNF-α production by human monocytes and PBMCs (19–21). In human alveolar macrophages, serotonin inhibits IL-12 and TNF-α release, but it increases IL-10, NO, and PGE_2 production via $5HT_2$ receptors (22). In the case of dendritic cells, 5HT impairs GM-CSF/IL-4–driven human monocyte-derived dendritic cell (MDDC) differentiation by reducing costimulatory molecule and CD1a expression as well as MLR stimulatory activity while increasing CD14 levels (23) and IL-10 production through $5HT_1$ or $5HT_7$ receptors (23). Others have shown that 5HT is shuttled from dendritic cells to naive T lymphocytes as a means to modulate T cell activation, proliferation, and differentiation (24), and that it might be necessary for optimal macrophage accessory function (25). In the context of inflammatory pathologies, 5HT regulates macrophage-mediated angiogenesis by reducing matrix metalloproteinase 12 (MMP12) expression in tumor-infiltrating macrophages (26), and its contribution to the development of pulmonary arterial hypertension depends on $5HT_{2B}$ receptor expression on bone marrow progenitors (27). Thus, 5HT modulates myeloid cell functions in a variable manner, and its effects are dependent on the profile of 5HT receptors expressed by each macrophage subtype. In this regard, previous studies have reported mRNA for $5HT_{1E}$, $5HT_{2A}$, $5HT_3$, $5HT_4$, and $5HT_7$ in human monocytes (21), $5HT_{1B}$, $5HT_{1E}$, and $5HT_{2B}$ in immature MDDC, $5HT_4$ and $5HT_7$ in mature MDDC (28), and $5HT_{2C}$ in murine alveolar macrophages (29).

The differential expression of 5HT receptors in macrophages at distinct states of functional polarization prompted an evaluation of the contribution of 5HT to macrophage polarization. We now report that 5HT stimulates human macrophage polarization toward the acquisition of an M2-like phenotype, and that this effect is mediated by $5HT_{2B}$ and $5HT_7$, which are preferentially expressed by anti-inflammatory M2 macrophages. These findings point to serotonin as a potential target for modulating macrophage polarization under physiological and pathological settings.

Materials and Methods

Generation of human monocyte-derived macrophages and cell isolation and culture

Human PBMC were isolated from buffy coats from normal donors over a Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient according to standard procedures. Monocytes were purified from PBMC by magnetic cell sorting using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes (>95% $CD14^+$ cells) were cultured at 0.5×10^6 cells/ml for 7 d in RPMI 1640 supplemented with 10% FCS (completed medium) at 37°C in a humidified atmosphere with 5% CO_2, and containing GM-CSF (1000 U/ml) or M-CSF (10 ng/ml) (both from ImmunoTools, Friesoythe, Germany) to generate M1(GM-CSF) and M2(M-CSF) monocyte-derived macrophages, respectively. Cytokines were added every 2 d. As previously described (3, 7, 8) (Gene Expression Omnibus, accession no. GSE27792; http://www.ncbi.nlm.nih.gov/geo/), the resulting macrophage populations exhibited a differential expression of the *STAB1*, *HTR2B*, *SERPINB2*, *COL23A1*, *THBS1*, *SERPINE1*, *MMP12*, *INHBA*, *CCR2*, *CD1B*, and *ALDH1A2* genes (Supplemental Fig. 1A) and differed in the protein levels of FOLR2, EGLN3, and DC-SIGN (30–32) (Supplemental Fig. 1B, 1C). Before treatment with 5HT, M2(M-CSF) macrophages were maintained in serum-free medium for 48 h, without a significant change in the level of expression of the polarization markers FOLR2, EGLN3, and DC-SIGN (Supplemental Fig. 1B, 1C). Macrophage activation was accomplished with LPS (*Escherichia coli* 055:B5, 10 ng/ml) for 24 h. For determination of LPS-induced cytokine expression, different doses of 5HT or the $5HT_{2B}$ agonist BW723C86 (Sigma-Aldrich) were added immediately before the addition of LPS. When receptor antagonists were used (SB204741, 1 μM; SB269970, 1 μM), they were added 1 h before 5HT and LPS addition.

RNA from human liver cells was obtained as previously described (33). Murine Kupffer cells, liver sinusoidal endothelial cells (LSEC), hepatocytes, and hepatic stellate cells (Ito cells) were isolated from 12-wk-old C57BL/6 mice using sequential pronase/collagenase digestion and a Nycodenz density-gradient centrifugation as described (34). Briefly, after perfusion via portal vein with 20 ml MEM (Life Technologies BRL), liver was digested with 10 ml DMEM/F-12 (Life Technologies BRL) containing pronase (0.5 mg/g body weight; Roche Diagnostics), followed by 10 ml of DMEM/F-12 containing 7 mg collagenase (Liberase Blendzymes; Roche Diagnostics). Digested liver was mashed ex vivo and incubated at 37°C for 25 min in 50 ml DMEM/F-12 solution containing 0.05% pronase and 20 μg/ml DNase I (Roche Diagnostics). The resulting suspension was filtered through a filter (mesh size 70 μm) and centrifuged over a 7.5% Nycodenz (Axis-Shield, Oslo, Norway) cushion at $1400 \times g$ for 15 min. The Ito cell–enriched fraction was recovered from the upper whitish layer. Kupffer and LSEC were simultaneously collected from the interface, and each cell type was further purified using anti-CD146 (for LSEC) and anti-CD11b (for Kupffer cells) microbeads (Miltenyi Biotec) according to the manufacturer's protocol. Purity of Ito cell preparations was assessed by autofluorescence 1 d after isolation. LPS (*E. coli* 055:B5) was purchased from Sigma-Aldrich, and IL-10 and IL-4 were obtained from BioLegend (San Diego, CA) and used at 50 ng/ml. The monoclonal anti-human IL-10 (R&D Systems, Abingdon, U.K.) blocking Ab was added at a final concentration of 2.5 μg/ml. The $5HT_{2B}$ receptor agonist BW723C86 and the antagonists SB204741 (for $5HT_{2B}$) and SB269970 (for $5HT_7$) were purchased from Sigma-Aldrich. Where indicated, SB204741 (1 μM) and SB269970 (1 μM) were added every 24 h during the process of in vitro macrophage generation using DMSO as a negative control.

ELISA

Macrophage supernatants were tested for the presence of cytokines and growth factors using commercially available ELISA for TNF-α, IL-10 (both from ImmunoTools), and IL-12p40 (OptEIA IL-12p40 set; BD Pharmingen, San Diego, CA), following the protocols supplied by the manufacturers.

Reporter gene assays

The luciferase-based plasmids CRE-Luc (containing 21 cAMP-response elements), SRE-Luc (containing three serum-response elements from the *c-FOS* promoter), and TRE-Luc (containing four copies of the canonical AP-1–binding site) were provided by Dr. W. Born (Departments of Orthopedic Surgery and Medicine, University of Zurich, Zurich, Switzerland). The TATA-pXP2 plasmid was derived from the promoterless pXP2 plasmid by insertion of the TATA-like sequence 5′-AGGGTATATAATGGAAG-3′ immediately upstream of the luciferase gene. The C/EBP-Luc plasmid, which contains four copies of the consensus C/EBP-binding element, was provided by Dr. Daniel G. Tenen (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA). CHO-K1 cells stably expressing the $5HT_{2B}$ receptor (ValiScreen; PerkinElmer, Boston, MA) were cultured in DMEM/F-12 with 1% dialyzed FCS and transiently transfected with 2 μg each reporter gene using SuperFect (Qiagen). After transfection, cells were cultured overnight and replated before exposure to 5HT (10^{-5} M) or BW723C86 (10^{-5} M) for 24 h. To normalize transfection efficiency, cells were cotransfected with an SV40 promoter-based β-galactosidase expression plasmid (RSV-βgal). Measurement of relative luciferase units and β-galactosidase activity were performed using a luciferase assay system (Promega) and a Galacto-Ligth kit (Tropix), respectively.

Quantitative real-time RT-PCR

Oligonucleotides for selected genes were designed according to the Universal ProbeLibrary system (Roche Diagnostics) for quantitative real-time PCR (qRT-PCR). Total RNA was extracted using an RNeasy kit (Qiagen), retrotranscribed, and amplified in triplicates. Results were expressed relative to the expression level of *GAPDH* RNA (human samples). When indicated, results were expressed relative to the mean of the expression levels of *GAPDH*, *TBP*, *HPRT1*, and *SDHA* RNA and normalized to the values obtained in untreated cells. In the case of RNA from murine samples, qRT-PCR was done using SYBR Green I detection for quantification of the level of expression of *Htr2B* (using oligonucleotides 5′-TGCCCTCTTGACAATCATGT-3′ and 5′-AGGGAAATGGCACAGAGATG-3′) and *Emr1* (5′-CCTGGACGAATCCTGTGAAG-3′ and 5′-GGTGGGACCACAGAGAGTTG-3′), as well as with *Tbp* gene levels (5′-GGGGAGCTGT-

GATGTGAAGT-3′ and 5′-CCAGGAAATAATTCTGGCTCA-3′) as a reference.

Western blot

Western blot was carried out on 10–100 μg cell lysates using standard procedures. Affinity-purified rabbit mAbs from Cell Signaling Technology (Danvers, MA) were used to detect phospho-ERK1/2 and phospho-MSK1. Detection of $5HT_{2B}$ was accomplished using a rabbit polyclonal antiserum (sc-25647; Santa Cruz Biotechonology), and protein loading control was done using mouse mAbs against human α-tubulin (B-7, sc-5286; Santa Cruz Biotechonology) or human vinculin (hVIN-1; Sigma-Aldrich).

Confocal microscopy and immunohistochemistry

Human melanoma tissues (s.c. tissue, lymph node, and lung metastasis) were obtained from patients with primary or metastatic lesions undergoing surgical treatment. Liver biopsies were obtained from patients undergoing surgery. In all cases, samples were obtained after written informed consent and following Medical Ethics Committee procedures. Thick sections (4 μm in depth) of cryopreserved tissue were first blocked for 10 min with 1% human Igs and then incubated for 1 h with either a rabbit polyclonal antiserum against human $5HT_{2B}$ (Santa Cruz Biotechnology), anti-CD163, anti–VE-cadherin or anti–HMB-45 mAbs, or isotype-matched control Abs. All primary Abs were used at 1–5 μg/ml, followed by incubation with FITC-labeled anti-mouse and Texas Red–labeled anti-rabbit secondary Abs. Samples were imaged with the ×63 PL-APO (numerical aperture, 1.3) immersion objective of a confocal scanning inverted AOBS/SP2 microscope (Leica Microsystems). Image processing and colocalization analyses (scatterplots) were assessed with Leica confocal software LCS-15.37. Normal human tissue microarrays were obtained from RayBiotech (Norcross, GA) and processed according to the manufacturer's recommendations using a Prestige affinity-purified polyclonal Ab against $5HT_{2B}$ (Sigma-Aldrich) and an anti-CD68 mAb (clone PG-M1, 1/100 dilution; DakoCytomation).

Statistical analysis

Statistical significance was assessed at the 0.05 level using a paired Student *t* test. Where indicated, a one-way ANOVA with Newman–Keuls for multiple comparison test was done.

Results

5HT receptors are differentially expressed by M1(GM-CSF) and M2(M-CSF) macrophages

HTR2B and *HTR7* genes code for the 5HT receptors $5HT_{2B}$ and $5HT_7$, respectively. Gene expression profiling on proinflammatory M1(GM-CSF) and anti-inflammatory M2(M-CSF) macrophages (GSE27792) (8) revealed that *HTR2B* and *HTR7* mRNA are preferentially expressed by M2(M-CSF) macrophages (Fig. 1A, 1B), and that *HTR7* is the only serotonin receptor–encoding gene expressed above background levels in M1(GM-CSF) macrophages (Fig. 1A, 1B). Analysis of five independent samples confirmed that *HTR2B* is expressed at significantly much higher levels in M2

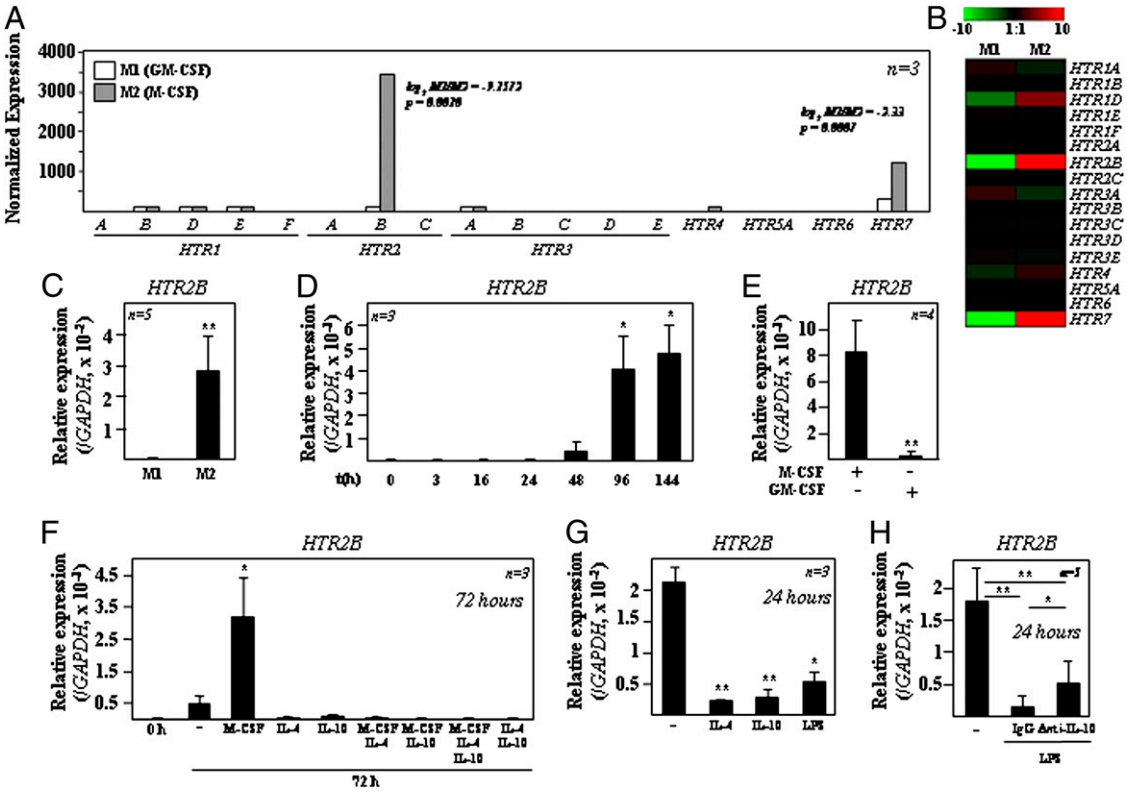

FIGURE 1. Preferential expression and cytokine responsiveness of HTR2B mRNA in human M2(M-CSF) macrophages. (**A**) Relative expression of the genes encoding serotonin receptors in M1(GM-CSF) and M2(M-CSF) macrophages, as determined by microarray DNA analysis (GSE27792). Shown data indicate the quantile-normalized fluorescence intensity of each probe. (**B**) Heat map representation of the data shown in (A). (**C**) *HTR2B* mRNA expression levels in M1(GM-CSF) and M2(M-CSF) macrophages as determined by qRT-PCR ($n = 5$). $^{**}p < 0.005$). (**D**) *HTR2B* mRNA expression levels along M2(M-CSF) macrophage polarization, as determined by qRT-PCR ($n = 3$). $^{*}p < 0.05$ compared with the level detected in monocytes ($t = 0$). (**E**) *HTR2B* mRNA expression levels in M2(M-CSF) macrophages after replacement of the culture supernatant with fresh complete medium containing either M-CSF or GM-CSF for 48 h ($n = 4$). $^{*}p < 0.05$. (**F**) *HTR2B* mRNA expression in monocytes exposed for 72 h to the indicated stimuli, as determined by qRT-PCR ($n = 3$). $^{*}p < 0.05$ compared with the level detected in monocytes maintained in RPMI 1640 (−). (**G**) *HTR2B* mRNA expression in M2(M-CSF) macrophages exposed for 24 h to the indicated stimuli, as determined by qRT-PCR ($n = 3$). $^{*}p < 0.05$ compared with the level detected in nonstimulated macrophages (−). (**H**) *HTR2B* mRNA expression in M2(M-CSF) macrophages exposed for 24 h to LPS in the presence of either a blocking anti–IL-10 or an isotype-matched (IgG) Ab, as determined by qRT-PCR ($n = 5$). $^{*}p < 0.05$, $^{**}p < 0.005$ after one-way ANOVA with Newman–Keuls multiple comparison test. In (C)–(H), results are presented as relative expression, which indicates *HTR2B* mRNA levels relative to *GAPDH* mRNA levels, and mean and SD are shown.

(M-CSF) than in M1(GM-CSF) macrophages (>500-fold) (Fig. 1C). Kinetic analysis showed that high *HTR2B* mRNA levels are first observed 48 h along M-CSF–driven polarization, reaching maximal levels after 96 h (Fig. 1D). Moreover, cytokine replacement experiments revealed that GM-CSF almost abrogated *HTR2B* mRNA levels from M2(M-CSF) macrophages (Fig. 1E). This inhibitory effect of GM-CSF prompted us to identify the cytokines affecting *HTR2B* expression at early time points. As shown in Fig. 1F, monocytes exposed to M-CSF for 72 h showed significantly higher levels of *HTR2B* mRNA than did non-stimulated monocytes, whereas other M2-polarizing cytokines (IL-4, IL-10) did not promote *HTR2B* mRNA expression but impaired the positive effects of M-CSF on the acquisition of *HTR2B* mRNA by monocytes. In fact, exposure of M2(M-CSF) macrophages to IL-4 or IL-10 for only 24 h led to a dramatic reduction in *HTR2B* mRNA levels, an effect also seen upon LPS stimulation (Fig. 1G). Because M2(M-CSF) macrophages respond to LPS by releasing large amounts of IL-10 (3, 8), we evaluated whether IL-10 mediated the LPS-induced downregulation of *HTR2B* mRNA expression. As shown in Fig. 1H, addition of a blocking anti–IL-10 Ab partly inhibited the action of LPS, indicating that IL-10 contributes to the inhibitory effect of LPS on *HTR2B* expression. Therefore, M2(M-CSF) macrophages are characterized by the presence of *HTR2B* mRNA, whose restricted expression is promoted by M-CSF. Moreover, M1(GM-CSF) macrophages generated in the presence of the Smad signaling inhibitor ALK4/5/7 exhibited an enhanced expression of *HTR2B* mRNA (data not shown), a result that agrees with the inhibitory action of the activin A/Smad signaling pathway on M2(M-CSF) macrophage polarization (8). Collectively, these results imply that *HTR2B* expression is upregulated by M-CSF but impaired by either IL-4, IL-10, GM-CSF, or ligands of ALK4/5/7.

$5HT_{2B}$ is expressed by M2-skewed tissue macrophages

To extend the above results, *HTR2B* gene expression in macrophages was assessed at the protein level. Immunocytochemical analysis confirmed the expression of $5HT_{2B}$ in M2(M-CSF) (Fig. 2A), and the differential expression of $5HT_{2B}$ was also seen by Western blot, which revealed a considerably higher expression in M2(M-CSF) than in M1(GM-CSF) macrophages (Fig. 2B). Next, we evaluated $5HT_{2B}$ expression in macrophages from normal and pathological tissues with a predominance of M2-skewed macro-

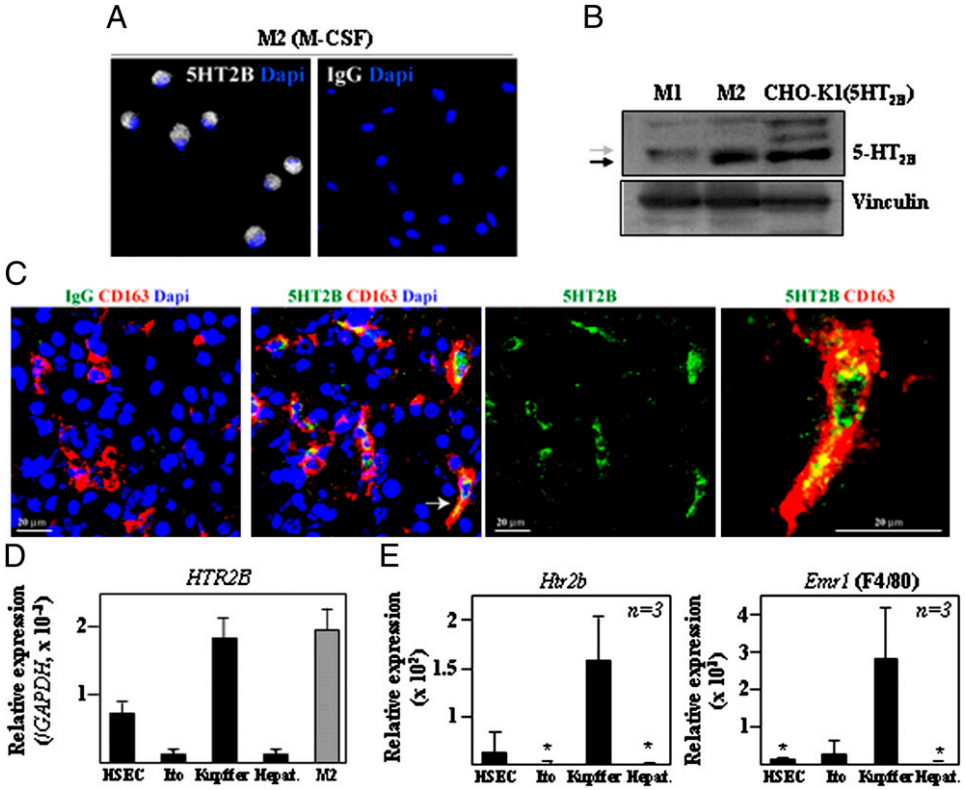

FIGURE 2. Expression of $5HT_{2B}$ and HTR2B mRNA in M2(M-CSF) and tissue-resident human macrophages. (**A**) Immunofluorescence analysis on M2 (M-CSF) macrophages, as determined by confocal microscopy (original magnification ×20) using a $5HT_{2B}$-specific rabbit polyclonal antiserum or non-specific rabbit antiserum (IgG). Nuclei were counterstained with DAPI (blue). The experiment was done on one preparation of in vitro–generated M2 macrophages. (**B**) Immunoblot analysis of $5HT_{2B}$ in lysates of $5HT_{2B}$-transfected CHO-K1 cells and M1(GM-CSF) and M2(M-CSF) macrophages. Vinculin protein levels were determined as a loading control. The light gray arrow indicates a nonspecific band, whereas the black arrow indicates the band corresponding to $5HT_{2B}$. One representative experiment out of three different donors and experiments is shown. (**C**) Confocal sections of human liver after double immunofluorescence analysis for $5HT_{2B}$ (green), a control rabbit antiserum (IgG, green), or the macrophage-specific marker CD163 (red). In the first two panels, nuclei were counterstained with DAPI (blue). The *right panel* shows a higher magnification of the macrophage indicated by an arrow in the second panel. Shown is a representative result of one out of three different sections from a human liver sample. (**D**) *HTR2B* mRNA expression in human sinusoidal endothelial cells (HSEC), Ito cells, Kupffer cells, and hepatocytes as determined by qRT-PCR. Results are expressed as relative expression, which indicates *HTR2B* the mRNA levels relative to *GAPDH* mRNA levels. As a reference, *HTR2B* mRNA levels in in vitro–generated human M2(M-CSF) macrophages were measured in parallel. Determinations were done in triplicate, and mean and SD are shown. (**E**) *Left panel*, *Htr2b* mRNA expression in murine sinusoidal endothelial cells (mSEC), Ito cells, Kupffer cells, and hepatocytes as determined by qRT-PCR. As a reference, *Emr1* (murine F4/80) mRNA levels were evaluated in the same populations (*right panel*). Results are expressed as relative expression, which indicates *Htr2b or Emr1* mRNA levels relative to the levels of *Tbp* mRNA, and mean and SD are shown ($n = 3$). *$p < 0.05$ compared with the level detected in Kupffer cells.

phages. Immunohistochemical analysis indicated the colocalization of $5HT_{2B}$ and the M2-associated marker CD163 in human liver Kupffer cells (Fig. 2C). Along the same line, the $5HT_{2B}$ reactivity in lung and colon tissue greatly resembled that of the macrophage marker CD68, further supporting the expression of this serotonin receptor in tissue-resident macrophages (Supplemental Fig. 2). In line with protein data, *HTR2B* mRNA was detected in isolated liver Kupffer cells, with expression levels similar to those found in in vitro–generated M2(M-CSF) macrophages (Fig. 2D), whereas *Htr2b* mRNA expression was preferentially found in murine Kupffer cells, which showed the highest expression of the myeloid marker *Emr1* (F4/80) (Fig. 2E). Taken together, these results demonstrate the expression of the $5HT_{2B}$-coding *HTR2B* gene in tissue-resident macrophages with anti-inflammatory ability (alveolar, colonic macrophages) (35, 36) or whose development is dependent on M-CSF (Kupffer cells) (37).

For evaluation of the $5HT_{2B}$ expression in macrophages from a pathological setting, we analyzed tumor-associated macrophages (TAM), whose M2-skewed polarization is well established (38, 39). Analysis of cutaneous primary melanomas revealed that $5HT_{2B}$ colocalizes with CD163, a macrophage marker preferentially found in M2-polarized macrophages (30, 40), and it showed a distribution pattern different from the melanoma-specific marker HMB45 (Fig. 3A, 3B). Most $5HT_{2B}$-positive macrophages were found in the proximity of VE-cadherin–positive endothelial cells (Fig. 3B). To confirm these results, the presence of *HTR2B* mRNA was analyzed in TAM isolated from carcinomas of various origins. Although to a lower extent than in vitro–generated M2(M-CSF) macrophages, *HTR2B* mRNA was detected in ex vivo–isolated TAM from gastric carcinoma, breast carcinoma, and melanoma (Fig. 3C). Therefore, and in agreement with its expression in M2 (M-CSF) macrophages, $5HT_{2B}$ constitutes a marker for macrophages exhibiting an M2-skewed polarization both in homeostatic and pathological conditions.

5HT and $5HT_{2B}$ receptor engagement trigger intracellular signaling and gene expression changes in M2(M-CSF) macrophages

To assess the functional expression of the $5HT_{2B}$ receptor in M2 (M-CSF) macrophages, we next evaluated the intracellular signals triggered in response to either 5HT stimulation or $5HT_{2B}$ engagement by the BW723C86 agonist. Screening of the activation state of 46 kinases revealed that ERK1/2 and its downstream targets MSK1/2 are phosphorylated in M2(M-CSF) macrophages in response to either 5HT stimulation or $5HT_{2B}$ ligation (data not shown). Kinetic analysis confirmed that both stimuli promote transient phosphorylation of ERK1/2 and MSK1 that peak at 10 min and diminished thereafter (Fig. 4A, 4B). Therefore, 5HT and BW723C86 activate ERK1/2 in M2(M-CSF) macrophages, confirming the presence of functional $5HT_{2B}$ receptors on their cell surface. These results are in agreement with previous results showing that ligation of the $5HT_{2B}$ receptor leads to ERK1/2

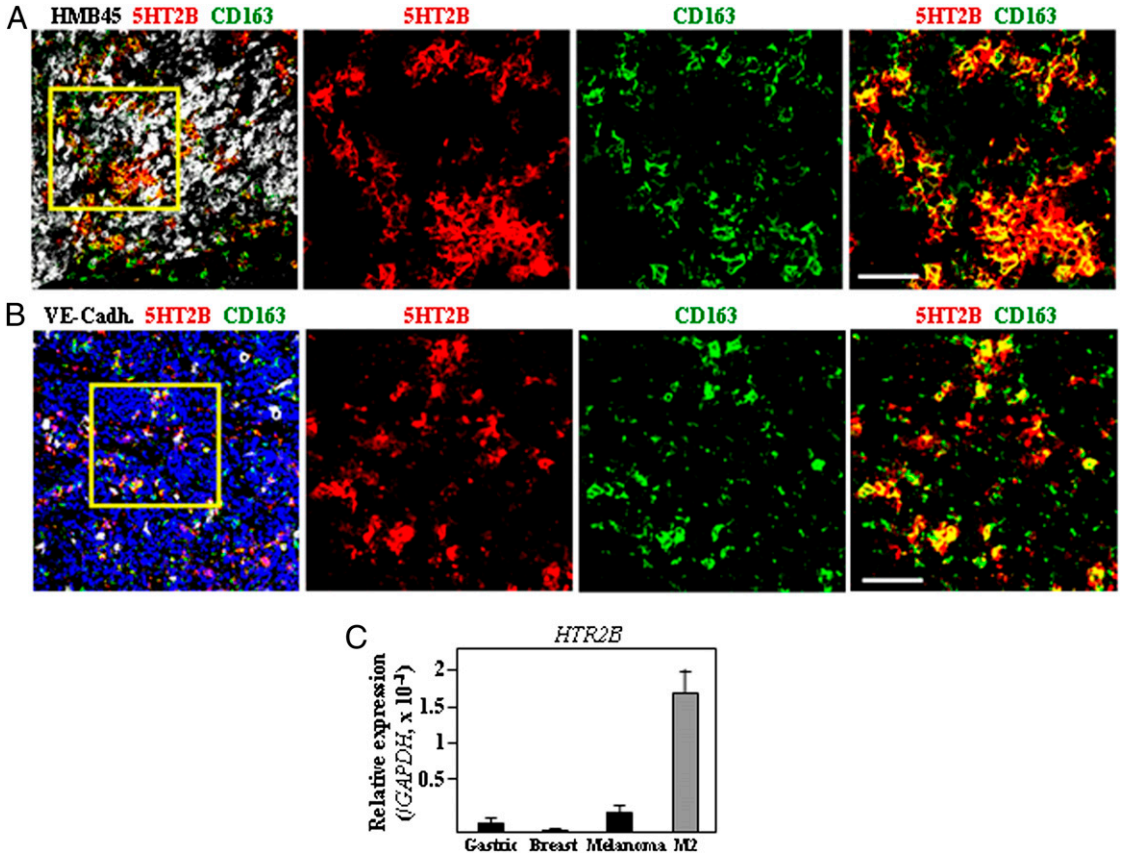

FIGURE 3. Expression of $5HT_{2B}$ in TAM from human melanoma. (**A** and **B**) Confocal sections of macrophages infiltrating a s.c. primary melanoma, as determined by immunofluorescence analysis of $5HT_{2B}$ (red), the macrophage marker CD163 (green), and either the melanoma marker HMB45 (white) (A) or the endothelial marker VE-cadherin (white) (B). Magnification of a $5HT_{2B}$/CD163 colocalizing area (indicated by the white rectangle) is shown in the *right panels*. In the *leftmost panel* in (B), nuclei were counterstained with DAPI (blue). In (A) and (B), representative results of one out of seven independent melanoma samples are shown. Scale bars, 50 μm. (**C**) *HTR2B* mRNA expression in ex vivo $CD14^+$ TAM isolated from one gastric carcinoma, one breast carcinoma, and one melanoma, as determined by qRT-PCR. For comparative purposes, *HTR2B* mRNA expression in a representative M2(M-CSF) macrophage preparation is shown. Results are expressed as relative expression, which indicates *HTR2B* mRNA levels relative to *GAPDH* mRNA levels.

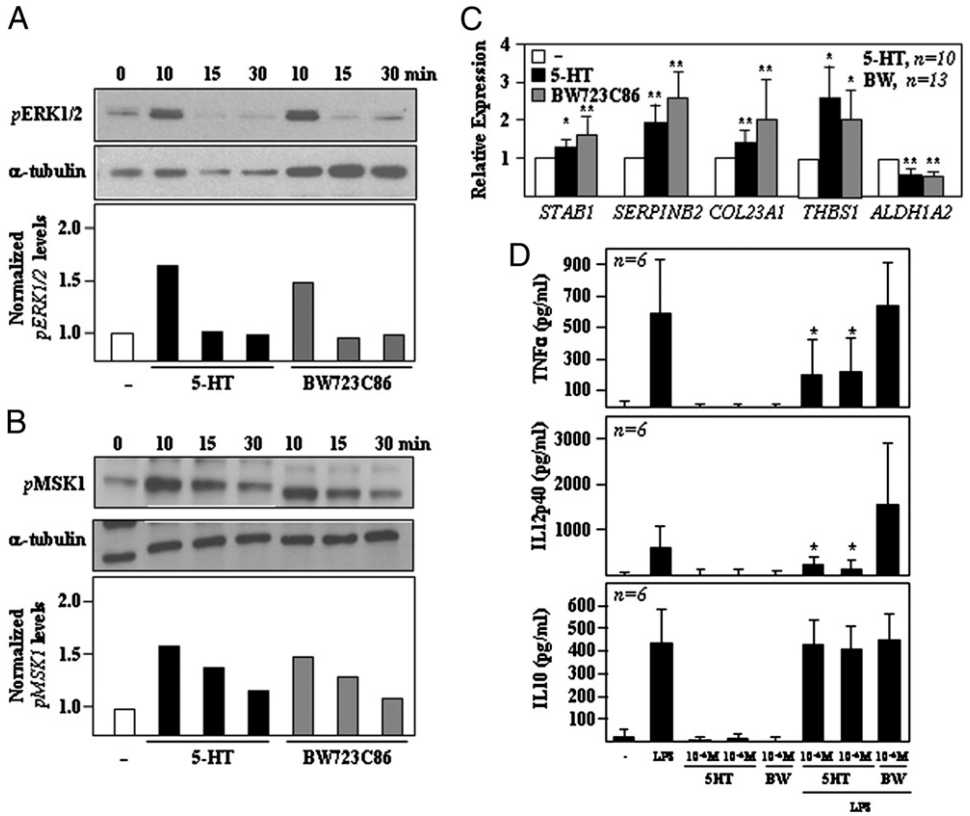

FIGURE 4. Effects of 5HT and the $5HT_{2B}$ agonist BW723C86 on intracellular signaling, polarization marker expression, and cytokine production. (**A**) ERK1/2 phosphorylation in lysates of untreated (−), 10^{-5} M 5HT-treated, or 10^{-5} M BW723C86-treated M2(M-CSF) macrophages, as determined by Western blot using Abs specific for total or phosphorylated ERK1/2 (*upper panels*). The relative level of phosphorylated ERK1/2 was determined after densitometric analysis and normalization with α-tubulin levels and is referred to the level of ERK1/2 phosphorylation in untreated cells (−) (*lower panel*). One representative experiment out of three different donors and experiments is shown. (**B**) MSK1 phosphorylation in lysates of untreated (−), 10^{-5} M 5HT-treated, or 10^{-5} M BW723C86-treated M2(M-CSF) macrophages, as determined by Western blot using Abs specific for phosphorylated MSK1 (*upper panels*). The relative level of phosphorylated MSK1 was determined after densitometric analysis and normalization with α-tubulin levels and is referred to the level of MSK1 phosphorylation in untreated cells (−) (*lower panel*). One representative experiment out of two different donors and experiments is shown. (**C**) ALDH1A2, COL23A1, STAB1, SERPINB2, and THBS1 mRNA expression levels in M2(M-CSF) macrophages exposed to either 5HT or BW723C86 (10^{-5} M) for 24 h, as determined by qRT-PCR. Results are expressed as relative expression (relative to TBP, HPRT1, SDHA, and GAPDH RNA levels) and are referred to the expression level observed in the absence of stimulation (−). Mean and SD of triplicate determinations on 10 (for 5HT) or 13 independent samples (for BW723C86) are shown. $*p < 0.05$, $**p < 0.005$. (**D**) ELISA determination of TNF-α, IL-12p40, and IL-10 levels in culture supernatants of M2(M-CSF) macrophages untreated or stimulated with LPS (10 ng/ml) for 24 h in the absence or presence of the indicated concentrations of 5HT or the $5HT_{2B}$ agonist BW723C86. Each determination was performed in triplicate on six independent samples, and means and SD are shown ($n = 6$). $*p < 0.05$ compared with the cytokine levels in LPS-treated macrophages.

activation in cardiomyocytes and hepatic stellate cells (41, 42), and they also fit with the ability of 5HT and BW723C86 to increase the activity of ERK1/2-regulated transcription factors that control macrophage activation and polarization (AP-1, C/EBP, SRF) (Supplemental Fig. 3).

The signaling capability of the $5HT_{2B}$ receptor in macrophages led us to explore whether 5HT or $5HT_{2B}$ ligation could modulate macrophage polarization. To that end, the expression of polarization-specific markers (8) was monitored in M2(M-CSF) macrophages that had been exposed to either 5HT or BW723C86 for 24 h. The expression of the M1(GM-CSF) polarization markers *ALDH1A2*, *CD1B*, and *MMP12* was significantly reduced by either 5HT or BW723C86 (Fig. 4C, Supplemental Fig. 4), whereas both agents enhanced the expression of the M2(M-CSF)–specific markers *SERPINB2*, *COL23A1*, *THBS1*, and *STAB1* (Fig. 4C). Besides, 5HT, but not the agonist, reduced the expression of the M1(GM-CSF)–specific markers *INHBA*, *CCR2*, and *SERPINE1* (Supplemental Fig. 4). These results are in agreement with the reported ability of 5HT to reduce the expression of MMP12 in murine macrophages (26), and they demonstrate that both 5HT and the $5HT_{2B}$ agonist BW723C86 influence the phenotypic macrophage polarization.

Because the defining difference between M1(GM-CSF) and M2(M-CSF) macrophages is their opposite cytokine profile upon stimulation, we next analyzed whether $5HT_{2B}$ ligation influenced the macrophage cytokine profile. To that end, M2(M-CSF) macrophages that had been maintained for 48 h in serum-free medium were exposed to LPS in the presence of 5HT and/or the $5HT_{2B}$ agonist. 5HT greatly reduced the LPS-stimulated production of TNF-α and IL-12p40, but it did not modify the LPS-stimulated production of IL-10 (Fig. 4D). In contrast, the presence of the $5HT_{2B}$ agonist BW723C86 had no significant effect on the LPS-stimulated release of TNFα, IL-12p40, or IL-10 (Fig. 4D). These results imply that 5HT modulates the functional polarization of human macrophages by limiting the production of proinflammatory cytokines, and that such a modulatory action is not mediated by the $5HT_{2B}$ receptor.

$5HT_7$ and $5HT_{2B}$ mediate the 5HT-dependent modulation of the macrophage gene expression profile

The distinct effects of 5HT and BW723C86 on gene expression (*INHBA*, *CCR2*, *SERPINE1*) (Supplemental Fig. 4) and on the LPS-stimulated cytokine profile (Fig. 4D) led us to evaluate

whether additional 5HT receptors might contribute to the macrophage polarization skewing ability of 5HT. As predicted from the gene expression profiling data (Fig. 1A, 1B), M2(M-CSF) macrophages also contained higher levels of *HTR7* mRNA than did M1(GM-CSF) macrophages (Fig. 5A). Similar to *HTR2B*, *HTR7* mRNA expression was detected at early time points (Fig. 5B) and inhibited by GM-CSF (Fig. 5C), IL-4, or LPS (Fig. 5D, 5E). However, and unlike *HTR2B*, *HTR7* mRNA expression was not significantly affected by M-CSF, IL-10 (Fig. 5D, 5E), or inhibition of the M-CSF–dependent ERK1/2 activation (data not shown). Therefore, we next determined whether $5HT_7$ contributed to the modulation of gene expression and cytokine profile promoted by 5HT. To that end, and owing to the lack of specific $5HT_7$ agonists, 5HT-mediated changes were monitored in the presence of the $5HT_7$ receptor–specific antagonist SB269970 and using the $5HT_{2B}$-specific antagonist SB204741 as a control. Whereas the $5HT_7$ antagonist SB269970 prevented the 5HT-induced upregulation of *STAB1*, *SERPINB2*, *THBS1*, and *COL23A1* gene expression (Fig. 6A), the $5HT_{2B}$ antagonist SB204741 only inhibited the positive effect of 5HT on *STAB1* and *SERPINB2* gene expression (Fig. 6A). Therefore, the modulation of the macrophage polarization phenotype caused by 5HT in M2(M-CSF) macrophages is mediated by $5HT_{2B}$ and $5HT_7$.

5HT abrogates proinflammatory cytokine production in macrophages through 5HT$_7$

Because $5HT_{2B}$ did not mediate the inhibitory action of 5HT on the LPS-stimulated release of IL-12p40 and TNF-α from M2(M-CSF) macrophages (Fig. 4D), the potential role of $5HT_7$ was tested. The presence of the $5HT_7$ antagonist SB269970 significantly reverted the inhibitory effect of 5HT on the LPS-stimulated production of both TNF-α and IL-12p40 (Fig. 6B). Conversely, in agreement with the results observed with the $5HT_{2B}$ agonist BW723C86, the $5HT_{2B}$ antagonist SB204741 did not affect the inhibitory effect of 5HT on the LPS-stimulated production of TNF-α and IL-12p40 from M2(M-CSF) macrophages that had been maintained for 48 h in serum-free medium (Fig. 6B). Because none of the antagonists modified the basal level of cytokine production (Fig. 6B), these results indicated that 5HT modulates the cytokine profile of LPS-stimulated human M2(M-CSF) macrophages primarily through interaction with the $5HT_7$ receptor.

5HT influences macrophage polarization

Given the ability of 5HT to alter phenotypic and functional macrophage polarization, we hypothesized that it might also contribute to the cytokine-dependent polarization of monocytes. To test this hypothesis, monocytes were subjected to M-CSF–driven polarization in the presence or absence of $5HT_{2B}$ or $5HT_7$ antagonists, and the expression of M1- and M2-specific markers was measured in the resulting monocyte-derived macrophage populations. Regarding M2(M-CSF) markers, the expression of *SERPINB2* and *HTR2B* mRNA was significantly diminished in the presence of the $5HT_{2B}$ antagonist SB204741, whereas *STAB1* and *THBS1* expression was significantly reduced in the presence of the $5HT_7$ antagonist SB269970 (Fig. 7A). Moreover, the simultaneous presence of both inhibitors limited the expression of *COL23A1* (Fig. 7A). Alternatively, the $5HT_{2B}$ antagonist SB204741 alone impaired the expression of the M1(GM-CSF) markers *ALDH1A2* and *CCR2* mRNA, whereas the combined presence of SB204741 and SB269970 reduced the expression of *MMP12* and enhanced that of *CD1B* mRNA (Fig. 7B). Collectively, these results demonstrate that antagonizing $5HT_{2B}$ or $5HT_7$ along the monocyte-to-macrophage differentiation process alters the pattern of expression of polarization markers, thus demonstrating that serotonin contributes to the acquisition of macrophage polarization markers during the M-CSF–driven monocyte-to-macrophage transition.

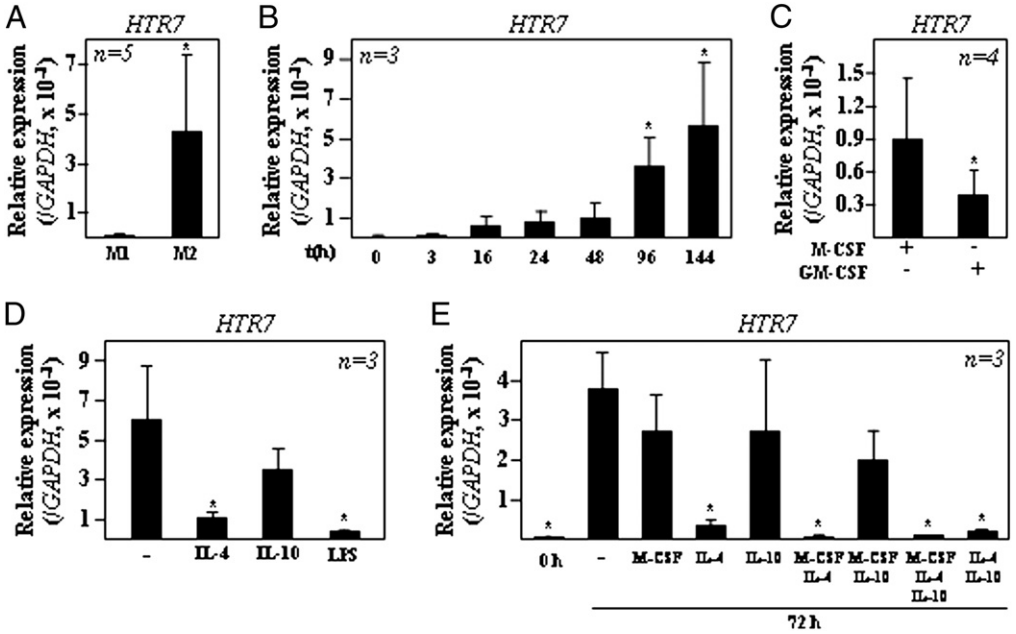

FIGURE 5. Regulated expression of HTR7 mRNA in human M2(M-CSF) macrophages. (**A**) *HTR7* mRNA expression levels in M1(GM-CSF) and M2 (M-CSF) macrophages as determined by qRT-PCR ($n = 5$). *$p < 0.05$. (**B**) *HTR7* mRNA expression levels along M2(M-CSF) macrophage polarization, as determined by qRT-PCR ($n = 3$). *$p < 0.05$ compared with the level detected in monocytes ($t = 0$). (**C**) *HTR7* mRNA expression levels in M2(M-CSF) macrophages after replacement of the culture supernatant with fresh complete medium containing either M-CSF or GM-CSF for 48 h ($n = 4$). *$p < 0.05$. (**D**) *HTR7* mRNA expression in M2(M-CSF) macrophages exposed for 24 h to the indicated stimuli, as determined by qRT-PCR ($n = 3$). *$p < 0.05$ compared with the level detected in nonstimulated macrophages (−). (**E**) *HTR7* mRNA expression in monocytes exposed for 72 h to the indicated stimuli, as determined by qRT-PCR ($n = 3$). *$p < 0.05$ compared with the level detected in monocytes maintained in RPMI (−). In (A)–(E), results are presented as relative expression, which indicates *HTR2B* mRNA levels relative to *GAPDH* mRNA levels, and mean and SD are shown.

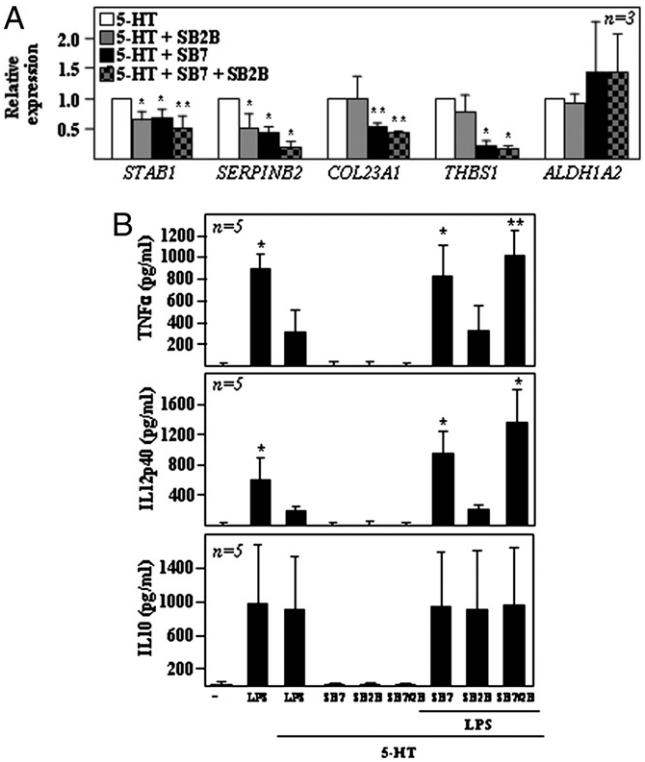

FIGURE 6. Effects of 5HT receptor antagonists on the 5HT-dependent changes in the gene expression and LPS-induced cytokine profile of M2 (M-CSF) macrophages. (**A**) STAB1, SERPINB2, COL23A1, THBS1, and ALDH1A2 mRNA expression levels in M2(M-CSF) macrophages exposed for 24 h to 5HT (10^{-5} M) in the absence or in the presence of SB269970 (SB-7), SB204741 (SB-2B), or both, as determined by qRT-PCR. Results are expressed as relative expression and are referred to the expression level of each gene in the presence of 5HT. Mean and SD of triplicate determinations on three independent samples are shown. $*p < 0.05$, $**p < 0.005$ compared with the level of expression detected in the absence of antagonists. (**B**) ELISA determination of TNF-α, IL-12p40, and IL-10 levels in culture supernatants of M2(M-CSF) macrophages untreated or stimulated with LPS (10 ng/ml) for 24 h in the absence or presence of 5HT, or 5HT plus the $5HT_7$ antagonist SB269970 (SB-7), the $5HT_{2B}$ antagonist SB204741 (SB-2B), or both antagonists. Each determination was performed in triplicate on five independent samples, and mean and SD are shown. $*p < 0.05$, $**p < 0.005$ compared with the level of each cytokine in macrophages exposed to LPS plus 5HT.

Discussion

Although 5HT controls important functions in the CNS, recent advances have now shown that 5HT functions as a regulator of cell proliferation as well as inflammation, tissue regeneration, and repair (12, 42, 43). The importance of macrophage polarization for an adequate regulation of these latter processes justifies the presence of functional 5HT receptors on their cell membrane and the 5HT ability to modulate macrophage effector functions. However, 5HT effects on the immune system are pleiotropic and incompletely understood (14). In the case of myeloid cells, 5HT variably affects the production of proinflammatory and anti-inflammatory cytokines from monocytes, macrophages, and dendritic cells, mainly because of the existence of a large set of 5HT receptor subtypes that exhibit cell- and tissue-specific patterns of expression (44). In the present study we provide evidence for the preferential expression of $5HT_{2B}$ in anti-inflammatory macrophages and liver Kupffer cells, demonstrate the effects of 5HT on the phenotypic and functional human macrophage polarization, and identify $5HT_{2B}$ and $5HT_7$ as the serotonin receptors that mediate the polarization skewing action of 5HT at the gene expression ($5HT_{2B}$ and $5HT_7$) and functional ($5HT_7$) levels.

Kupffer cells represent >50% of resident macrophages in the whole body, and their development and maturation are dependent on M-CSF (37). Because of their location in the liver sinusoids, Kupffer cells are continuously exposed to the 5HT levels found in peripheral blood after its release from either enterochromaffin cells (under physiological conditions) or platelets (after degranulation events). Therefore, it is conceivable that $5HT_{2B}$ might contribute to adjusting the activation state of Kupffer cells to extracellular 5HT levels. Additionally, because $5HT_{2A}$ and $5HT_{2B}$ mediate serotonin-dependent liver regeneration (12), a process where Kupffer cell–derived cytokines also participate (45), it is tempting to speculate that the presence of $5HT_{2B}$ on Kupffer cells has a direct impact on liver regeneration. In this regard, the ability of the $5HT_{2B}$ agonist BW723C86 to favor the in vitro maintenance of the M2 macrophage polarization state, characterized by its tissue repair– and cell growth–promoting properties, supports such a hypothesis.

Besides Kupffer cells, we have identified $5HT_{2B}$ expression in TAM, which display a tumor-promoting and immunosuppressive M2-like polarization (46). A link between 5HT and macrophages in cancer progression has been already established, as 5HT enhances angiogenesis in murine colon cancer allografts via the 5HT-dependent reduction of MMP12 expression by tumor-infiltrating macrophages (26). Our results on the phenotypic changes induced by 5HT and BW723C86 on human macrophages are compatible with these findings because: 1) both stimuli inhibit *MMP12* mRNA expression (Supplemental Fig. 4), thus suggesting $5HT_{2B}$ as a mediator of inhibitory effect of 5HT on MMP12 expression; and 2) both stimuli skew macrophages toward M2 polarization, which is associated with potent proangiogenic activity (46). Therefore, our results suggest that $5HT_{2B}$ is a relevant receptor whose ex-

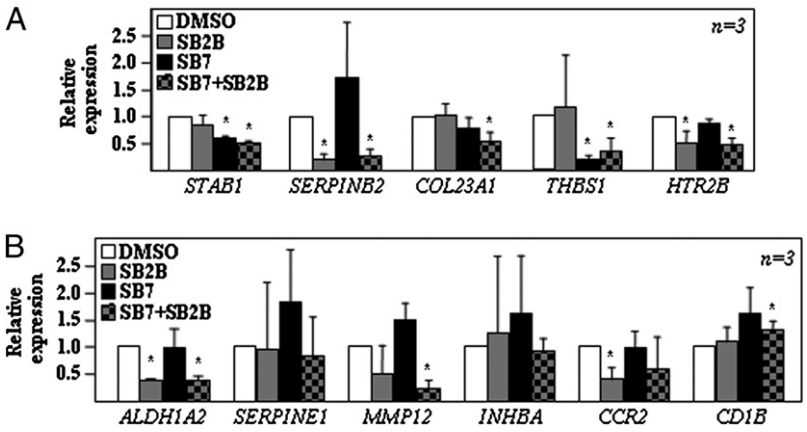

FIGURE 7. The presence of $5HT_{2B}$ or $5HT_7$ antagonists during the monocyte-to-macrophage differentiation process impairs the acquisition of macrophage polarization markers. Expression levels are shown of STAB1, SERPINB2, COL23A1, THBS1, and HTR2B (**A**) and ALDH1A2, SERPINE1, MMP12, INHBA, CCR2, and CD1B (**B**) mRNA in M2(M-CSF) macrophages generated in the continuous presence of DMSO, SB269970 (SB7), SB204741 (SB2B), or both, as determined by qRT-PCR. Results are expressed as relative expression (relative to GAPDH RNA levels) and are referred to the expression level of each gene in the presence of DMSO. Mean and SD of triplicate determinations on three independent samples are shown. $*p < 0.05$ compared with the level of expression detected in the absence of antagonists.

pression on human macrophages might mediate the proproliferative and tissue repair activity of 5HT in peripheral tissues.

Alternatively, the location of $5HT_{2B}$-expressing TAM in the proximity of VE-cadherin–positive endothelial cells (Fig. 3) might be also of significance considering the link between $5HT_{2B}$ expression and pulmonary arterial hypertension (PAH) (47). PAH is characterized by vascular remodeling secondary to abnormal cell proliferation and extracellular matrix deposition, with the latter two activities commonly linked to M2 macrophage polarization. Thus, the presence of $5HT_{2B}$ in lung macrophages, as well as its expression in human TAM, which promote tumor cell growth and dissemination, raises the question of whether $5HT_{2B}$-positive macrophages might contribute to PAH by enhancing proliferation of other cell types (endothelium, smooth muscle) or through exacerbated extracellular matrix deposition. This possibility is compatible with the recent finding that PAH development requires the expression of the murine $5HT_{2B}$ receptor in bone marrow–derived progenitor cells (27).

Previous reports have documented the ability of 5HT to modulate the production of numerous cytokines in mononuclear cells, monocytes, monocyte-derived dendritic cells, and ex vivo–isolated macrophages (14, 48). Whereas the inhibition of TNF-α production is a general finding, the effects of 5HT on other proinflammatory cytokines and IL-10 are variable, and they appear to be cell type–dependent. Interestingly, most previous studies have evaluated 5HT on myeloid cells in medium containing serum, where 5HT concentration is as high as 1–2 μM, thus potentially leading to an underestimation of the influence of 5HT. Alternatively, the presence of serum factors could modulate or synergize with 5HT, and it might have caused an overestimation of the 5HT influence. In an effort to avoid these potentially confounding issues, we have carried out all experiments on macrophages maintained in serum-free medium for 48 h. Thus, apart from the cell type–specific nature of 5HT actions, the distinct medium used for assaying 5HT might explain the discrepancy between the lack of effect of 5HT on IL-10 release seen in our experiments and those of others (20, 49) and the positive action of 5HT in IL-10 production described in human alveolar cells and monocyte-derived dendritic cells (22, 23). Despite these discrepancies, our results are in agreement with the lack of effect of $5HT_2$ agonists on the LPS-induced monocyte cytokine production (21).

The results presented in this study reveal that the modulation of the phenotypic and functional macrophage polarization by 5HT is mediated by both $5HT_{2B}$ and $5HT_7$ receptors. At the functional level, our results point to $5HT_7$ as the critical receptor mediating the effect of 5HT on macrophage cytokine production. Within the myeloid cell lineage, *HTR7* mRNA has been identified in human monocytes (21) and mature MDDC (28), and, similar to the case of M2(M-CSF) macrophages, $5HT_7$ has been shown to negatively regulate the secretion of TNF-α from mature MDDC (28) and LPS-activated monocytes (21). Whereas $5HT_7$ agonists also enhance the release of IL-1β and IL-8 from MDDC (28) and monocytes (21), opposite activities on IL-12 have been reported in both cell types. Our results extend those previous findings to the case of anti-inflammatory macrophages and, to our knowledge, also provide the first evidence that $5HT_7$ directly contributes to the gene expression changes provoked by 5HT in human macrophages.

The ability of 5HT to promote the maintenance of the M2 phenotype and to impair the release of proinflammatory cytokines is in agreement with the recent finding that platelet-derived 5HT delays activated virus-specific $CD8^+$ T cell infiltration in a murine model of noncytopathic lymphocytic choriomeningitis viral infection (50), and suggests that tissue macrophages might overcome the action of peripheral 5HT to display their full range of proinflammatory activities. In fact, note that 5HT is released from enterochromaffin cells along the gut, where macrophages mostly release anti-inflammatory molecules (including IL-10) upon stimulation (36). 5HT might therefore contribute to the maintenance of the anti-inflammatory profile of lamina propria macrophages, an effect that could be mediated by $5HT_{2B}$ on intestinal macrophages (Supplemental Fig. 2). This possibility, combined with the functions of 5HT in the hepato-gastrointestinal tract (9), should prompt further investigation on the role of the macrophage $5HT_{2B}$ and $5HT_7$ receptors in liver and gut physiopathology.

Disclosures

The authors have no financial conflicts of interest.

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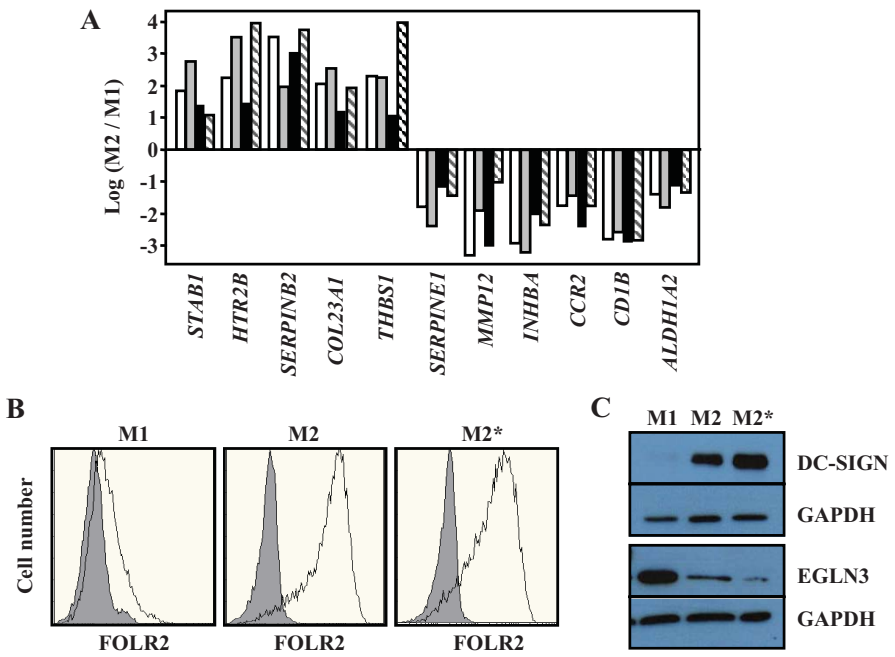
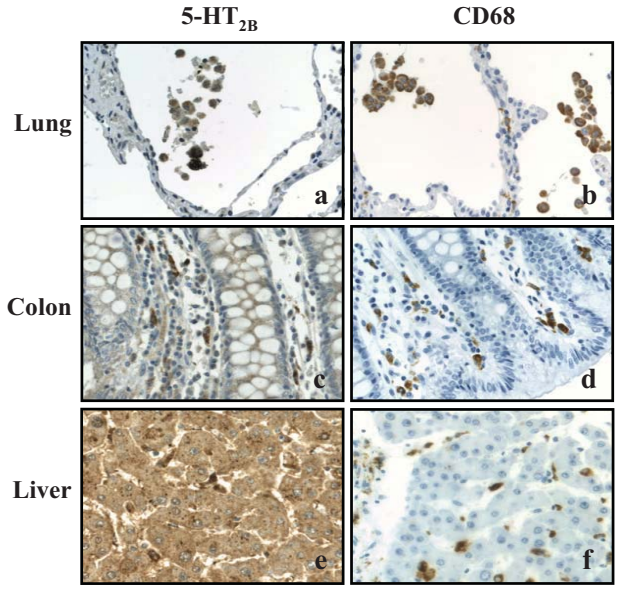
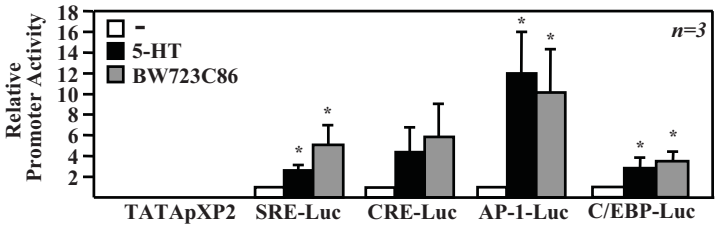


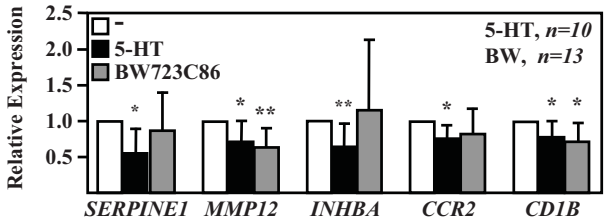
Supplementary Figure 1.- **A**.- Relative mRNA expression levels of the indicated genes in four independent M1(GM-CSF) and M2(M-CSF) macrophage samples, as determined by qRT-PCR. Results are shown as the log of the ratio between the expression of each mRNA in M2(M-CSF) and M1(GM-CSF) macrophages. **B,C**.- Expression of FOLR2 (**B**), DC-SIGN (**C**) and EGLN3 (**C**) in M1(GM-CSF), M2(M-CSF) and M2(M-CSF) macrophages that had been maintained under serum-free conditions for 48 hours (M2*), as determined by flow cytometry with a FOLR2-specific polyclonal antiserum (**B**) or Western blot using polyclonal antisera specific for either DC-SIGN or human PHD3 (**C**). In **C**, and for loading control purposes, the levels of GAPDH were determined with a monoclonal antibody against GAPDH (sc-32233; Santa Cruz Biotechnology, Santa Cruz, CA). In B-C, shown are the representative results of one out of two independent experiments.



Supplementary Figure 2.- Expression of 5-HT2B in human tissues, as determined by immunohistochemistry on lung (a,b), colon (c,d) and liver (e,f) tissue sections. Shown are light microscopy images (40X magnification) of the indicated tissues after staining with a anti-5-HT2B antibody rabbit polyclonal (a,c,e) or an anti-CD68 murine monoclonal antibody (b,d,f). Similar results were obtained on two independent samples of each tissue, and one of them is shown.



Supplementary Figure 3.- Activity of the indicated reporter constructs in CHO-K1 ($5\text{-}HT_{2B}$) stable transfectants either unstimulated (-) or exposed to 5-HT or BW723C86 (10^{-5} M). For normalization, cells were co-transfected with the RSV-β-gal expression plasmid, and results are presented as Relative Promoter Activity, that indicates the units of luciferase activity per unit of β-galactosidase activity for each assay condition. Three independent experiments were done, and evaluation of luciferase activity was done in triplicate. Shown are the mean and standard deviations of the three experiments (*, $p < 0.05$).



Supplementary Figure 4.- Effects of 5-HT and BW723C86 on polarization marker expression.- *SERPINE1, MMP12, INHBA, CCR2* and *CD1B* mRNA expression levels in M2(M-CSF) macrophages exposed to either 5-HT or BW723C86 (10^{-5} M) for 24 hours, as determined by qRT-PCR. Results are expressed as Relative Expression (relative to *TBP*, *HPRT1*, *SDHA* and *GAPDH* RNA levels) and referred to the expression level observed in the absence of stimulation (-). Mean and standard deviation of triplicate determinations on ten (for 5-HT) or thirteen independent samples (for BW723C86) are shown (*, $p < 0.05$; **, $p < 0.005$).

Serotonin-dependent gene expression profile in human macrophages.

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Running title :Serotonin-dependent macrophage gene profile

ABSTRACT

Serotonin (5-hydroxytryptamine, 5HT) is a monoamine neurotransmitter that regulates mood, appetite and sleep within the nervous system. In periphery, however, 5HT acts as a growth factor and a regulator of wound healing and inflammation. Since 5HT skews human macrophage polarization via engagement of 5-HT_{2B} and 5-HT_7 receptors, we undertook the identification of the 5HT-dependent gene expression profile in human macrophages. We now report that the 5-HT_7-cAMP-PKA axis is responsible for the 5HT-dependent expression of genes encoding growth factors (EREG), growth factor receptors (MET), cytokines (IL1B) and cell surface molecules involved in macrophage activation (TREM1), all of which might contribute to the growth-promoting and immunomodulatory functions of 5HT. In addition, we have determined that 5HT, via ligation of 5-HT2B, regulates the expression of type I IFN-responsive genes (CXCL10, IFI2, IFIT3, TNFSF18), thus defining a novel link between 5HT and the expression of genes that govern antiviral responses by human macrophages.

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5HT) is a monoamine neurotransmitter derived from L-tryptophan via a rate-limiting reaction catalyzed by two tryptophan hydroxylases (TPH1, THP2) with different expression patterns (TPH1 in periphery, TPH2 in brain) (1). Serotonergic neurons regulate mood, appetite, sleep, memory and learning, and modulation of 5HT at synapses is a major mechanism of action of several pharmacological antidepressants (2-4). However, ninety per cent of the human body's 5HT is synthesized and secreted by the enterochromaffin cells in the gastrointestinal tract, where 5HT controls intestinal movements. Enterochromaffin cell-derived 5HT is actively taken up by blood platelets that, upon activation, release it to exert a vasoconstrictor and growth factor function on smooth muscle cells (5), hepatocytes (6) and endothelial cells (7), thus contributing wound healing. 5HT also functions as a regulator of inflammation (8), as it modulates cytokine production in a cell type-dependent manner (9-11). In human alveolar macrophages, 5HT inhibits IL-12 and TNFα release, but increases IL-10, NO and PGE2 production (12), whereas it is capable of modulating T-cell activation, proliferation and differentiation (13). Regarding inflammatory pathologies, 5HT regulates macrophage-mediated angiogenesis by reducing MMP12 expression (14), and contributes to Pulmonary Arterial Hypertension (PAH) via 5-HT_{2B} receptor engagement on bone-marrow progenitors (15). 5HT signals through seven types of receptors (5-HT_{1-7}), six of which belong to the G protein-coupled superfamily of receptors (5-HT_1, 5-HT_2, 5-HT_4, 5-HT_5, 5-HT_6, 5-HT_7).

GM-CSF and M-CSF promote the *in vitro* differentiation of macrophages with distinct morphology, pathogen susceptibility and inflammatory function (16-18). GM-CSF drives the generation of monocyte-derived macrophages that produce pro-inflammatory cytokines in response to LPS and display high antigen-presenting and tumoricidal capacity (M1-polarized macrophages). Conversely, M-CSF yields macrophages that release IL-10 in response to pathogens and exhibit high phagocytic and pro-tumoral activity (M2-polarized macrophages) (16,19). We have previously demonstrated that the complement of 5HT receptors is different between pro-inflammatory (M1) and anti-inflammatory (M2) human macrophages, and that the presence of 5-HT_{2B} and 5-HT_7 shapes the phenotype and effector functions of monocyte-derived macrophages (20). We now report the identification of the 5HT-dependent gene expression profile of human macrophages, demonstrate that the 5-HT_7-cAMP-PKA signaling axis is critical for the 5HT-dependent acquisition of anti-inflammatory functions, and provide evidences for the ability of 5HT to alter the expression of IFN-responsive genes via engagement of 5-HT_{2B}. Our findings establish 5HT-initiated signaling cascades as critical modulators of macrophage polarization and potential targets for modulating inflammatory responses.

MATERIALS AND METHODS

Generation of human monocyte-derived macrophages and cell isolation and culture.- Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats from normal donors over a Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient according to standard procedures. Monocytes were purified from PBMC by magnetic cell sorting using CD14 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). Monocytes (>95% CD14+ cells) were cultured at 0.5 x 10^6 cells/ml for 7 days in RPMI supplemented with 10% fetal calf serum (FCS) (completed medium), at 37ºC in a humidified atmosphere with 5% CO_2, and containing GM-CSF (1000U/ml) or M-CSF (10 ng/ml) (both from ImmunoTools GmbH, Friesoythe, Germany) to generate M1(GM-CSF) (hereafter termed M1) and M2(M-CSF) (hereafter termed M2) monocyte-derived macrophages, respectively. Cytokines were added every two days. Before treatment with 5HT, M2 macrophages were maintained in serum-free medium for 48 hours, without a significant change in the level of expression of M2 polarization-specific markers. Macrophage activation was accomplished with LPS (E. coli 055:B5, 10 ng/ml) for 24 hours. Receptor antagonists SB204741 (for 5-HT_{2B}) and SB269970 (for 5-HT_7) were purchased from Sigma, used at 1 μM, and added 1 hour before 5HT treatment. 5HT Receptor-specific agonists included BW723C86 (5-HT2B) and LP12 (5-HT7) where used at 10-5M and 10-6M respectively. In the case of cAMP analogs the concentration used was 200 μM for Br-cAMP, 50μM for dBR-cAMP, and 10 μM in the case of

forskolin. The PKA-specific activator 6-BNZ-cAMP and the Epac-specific activator 8-pCPT-2′-O-Me-cAMP were obtained from Sigma Aldrich and used at 200 μM and 100 μM, respectively. The PKA-specific inhibitor RP-8-CPT-cAMPS was obtained from Biolog and was used at 1 μM.

ELISA.- Macrophage supernatants were tested for the presence of cytokines and growth factors using commercially available ELISA for TNF-α, IL-10, IL1B (all from Biolegend), IL-12 p40 (OptEIA™ IL-12 p40 set, BD Pharmingen, San Diego, CA) and EREG (USCN life science Inc.), following the protocols supplied by the manufacturers.

Quantitative real-time RT-PCR.- Oligonucleotides for selected genes were designed according to the Universal Probe Roche library system (Roche Diagnostics) for quantitative real time PCR (qRT-PCR). Total RNA was extracted using the RNeasy kit (Qiagen), retrotranscribed, and amplified in triplicates. Results were expressed relative to the expression level of GAPDH RNA to the values obtained in untreated cells.

Microarray analysis.- RNA was isolated from three independent preparations of macrophages exposed to 5HT, BW723C86 or DMSO for 6 hours by using AllPrep DNA/RNA/Protein Mini Kit (Qiagen). Labeled RNA was used as a hybridization probe on Whole Human Genome Microarrays (Agilent Technologies, Palo Alto, CA). Gene expression data were filtered eliminating any non probe-gene and probes with low signals and questionable quality. A total of 36232 useful probes were obtained for subsequent analysis, and quantiles were normalized. For the detection of differentially expressed genes, a linear model was fitted to the data and empirical Bayes moderated statistics were calculated using the limma package from Bioconductor (Smyth, G. K. (2005). Limma: linear models for microarray data. In: 'Bioinformatics and Computational Biology Solutions using R and Bioconductor'. R. Gentleman, V. Carey, S. Dudoit, R. Irizarry, W. Huber (eds), Springer, New York, pages 397-420). All computations were done using R statistical software.

Statistical analysis.- Statistical significance was assessed at the 0.05 level using a paired Student t-test.

RESULTS

Serotonin inhibits proinflammatory cytokine production in human macrophages.- We have previously demonstrated that 5HT inhibits the LPS-induced production of IL-12p40 and TNFα and had no effect on IL-10 production when both stimuli (5HT and LPS) are added simultaneously. However, it remained to be determined whether 5HT-pretreated macrophages exhibited an altered proinflammatory cytokine release. To that end, macrophages were pretreated with 5HT and exposed to LPS after 6 hours. As seen in *Figure 1A*, 5HT pretreatment led to a significant reduction in the LPS-induced production of IL-12p40 and TNFα, whereas it had no effect on IL-10 production. Therefore, 5HT conditions macrophages for diminished production of proinflammatory cytokines upon exposure to a pathogenic stimulus.

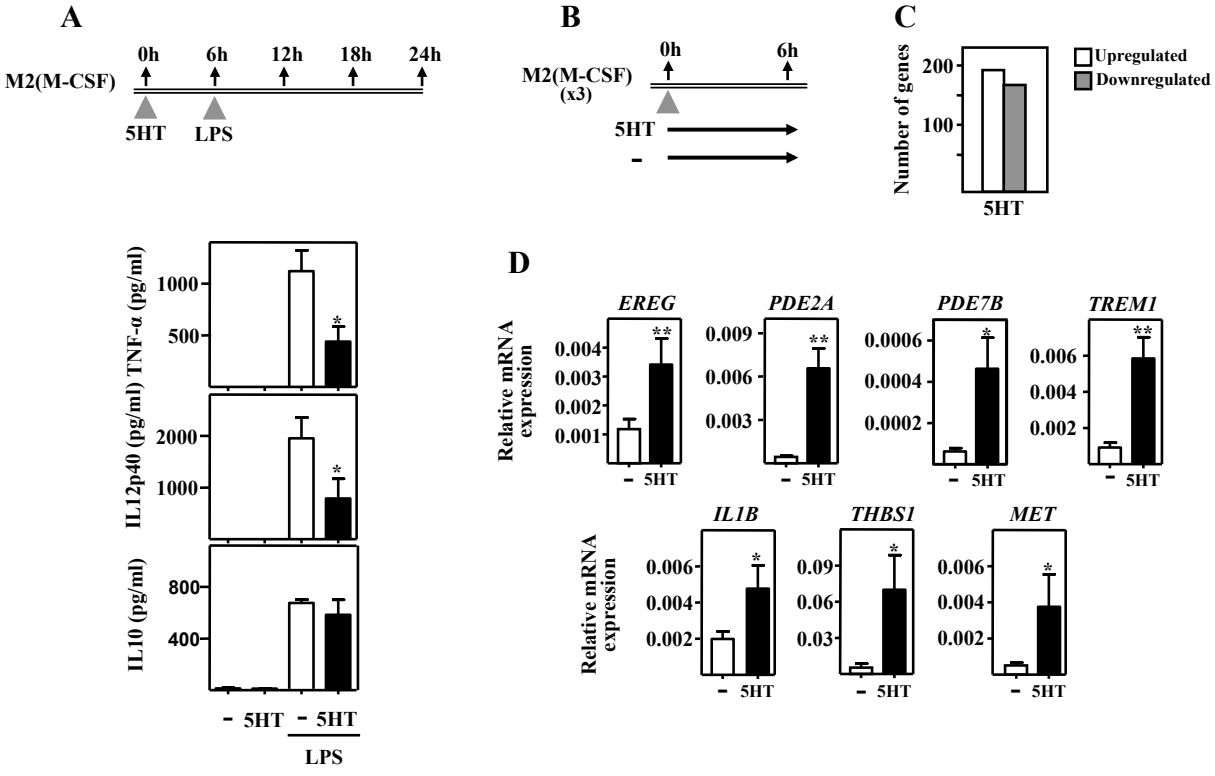

Figure 1.- Serotonin shapes the cytokine and gene expression profile of human macrophages.- A. Determination of TNFα, IL-12p40 and IL-10 levels in culture supernatants of non-treated (-) or 5HT-treated (6 h) macrophages, after stimulation with LPS (10 ng/ml) for 18 hours. Each determination was performed in triplicate on three independent macrophage samples, and means and standard deviations are shown (n=4, *, $p < 0.05$ compared to the cytokine levels in LPS-treated macrophages (LPS)). **B**. Schematic representation of the profiling experiments, with indication of the treatment and time points for sample collection. **C**. Quantification of the number of genes whose expression is significantly upregulated or downregulated (adjusted $p<0.05$) after treatment with 5HT for 6 hours. **D**. Relative expression of the indicated genes in non-treated (-) or 5HT-treated (6 h) macrophages. Results are expressed as Relative Expression that indicates the expression of the corresponding mRNA relative to the GAPDH mRNA levels in each sample. Three independent experiments were performed and mean and standard deviations are shown (*, $p<0.05$; **, $p<0.01$).

Identification of the 5HT-dependent gene expression profile in human macrophages.- As a mean to determine the 5HT-dependent transcriptome in human macrophages, cells were exposed to 5HT and global gene expression changes analyzed after 6 hours (*Figure 1B)*. Transcriptional profiling revealed that the expression of 361 genes was significantly altered upon exposure to 5HT, with a roughly similar amount of upregulated and downregulated genes (*Figure 1C*). Analysis of 5HT-responsive genes revealed the presence of numerous genes associated with macrophage activation, including *IL1B* and *TREM1*, as well as genes that code for proteins that control intracellular cAMP levels like *PDE2A* and *PDE7B* (*Table I*). To validate the microarray transcriptomic data, 5HT-dependent gene expression was evaluated in independent macrophage samples. As shown in *Figure 1D*, 5HT significantly upregulated the expression of genes like *PDE2A, PDE7B, EREG, ILIB, TREM1, THBS1* and *MET*. Assessment of the functional activity of 5HT-regulated genes indicated their involvement in modulation of cAMP-mediated signalling, VDR/RXR Activation, Docosahexaenoic Acid (DHA) Signalling, Clathrin-mediated Endocytosis Signalling and mTOR Signalling (*Supplementary Figure 1*), and predicted forskolin and PGE2 as their putative upstream regulators (*Supplementary Figure 2*). As a whole, these results confirmed the ability of 5HT to modulate the expression of genes directly involved in macrophage activation.

Top 5HT regulated genes						Top BW723C86 regulated genes					
Gene symbol	5HT/C	*p*	Gene symbol	5HT/C	*p*	Gene symbol	BW/C	*p*	Gene symbol	BW/C	*p*
PDE2A	13.20	2.9844E-05	*BNIPL*	-2.08	0.00529128	*EIF5B*	6.51	0.0037544	*HEPACAM*	-2.20	0.00293896
PDE7B	5.99	0.00130597	*TPTE2P1*	-2.08	0.04562586	*CISH*	4.80	0.00227339	*PLAC1*	-2.25	0.01054164
EIF5B	4.98	0.01414957	*MAP2*	-2.12	0.00786528	*REXO1L1*	4.64	0.01407177	*KAZALD1*	-2.25	0.00587898
MET	4.91	0.00497563	*ECEL1*	-2.12	0.04486986	*DDX6*	4.52	0.00636526	*IGSF10*	-2.27	0.00786169
TREM1	4.42	0.00122957	*CDC20B*	-2.12	0.00667189	*RSAD2*	4.48	0.00732694	*GCNT4*	-2.27	0.01770033
PLAT	4.34	0.01510161	*SPRR1A*	-2.13	0.00620383	*IFIT2*	4.45	0.00132356	*LALBA*	-2.34	0.04010939
AZI2	4.06	0.00547121	*GRIA1*	-2.17	0.03452025	*RPL21*	4.33	0.01810706	*CDC20B*	-2.34	0.00573081
STAT4	3.95	0.00074132	*NEK7*	-2.19	0.00973714	*NDUFA6*	4.18	0.00598053	*RSPH4A*	-2.35	0.00249602
EREG	3.27	0.01377387	*PLEKHG7*	-2.19	0.00708648	*AZI2*	3.89	0.00627127	*KPNA7*	-2.44	0.00157983
SIPA1L1	3.04	0.00128322	*KLK13*	-2.21	0.02991914	*CDC42*	3.82	0.00284724	*FAM135B*	-2.44	0.00864682
SIDT1	3.03	0.00232599	*MCHR2*	-2.25	0.01261128	*IMPACT*	3.82	0.01943155	*OR4K2*	-2.46	0.02915229
HOXB3	2.99	0.02514732	*HDAC9*	-2.25	0.02328635	*OASL*	3.69	0.00083665	*TMPRSS15*	-2.49	0.01953383
SEMA5A	2.91	0.0015864	*TEX9*	-2.27	0.04291463	*CST5*	3.68	0.02976087	*ADAM12*	-2.52	0.00303682
ST6GALNAC3	2.90	0.00018912	*H2BFWT*	-2.31	0.00196815	*RGS4*	3.62	0.00263792	*ANKRD56*	-2.57	0.00216008
SYT14	2.79	0.03301681	*TMPRSS15*	-2.32	0.00292369	*EIF4B*	3.58	0.01384987	*YJEFN3*	-2.59	0.00951135
CDC42	2.75	0.01927368	*KPNA7*	-2.35	0.00462084	*PCDH9*	3.33	0.00714172	*TTN*	-2.62	0.00385423
BTBD11	2.65	0.00058571	*ADAMTSL1*	-2.46	0.03811199	*SLC9A3*	3.26	0.04843774	*WT1*	-2.63	0.00024227
CREM	2.65	0.00642774	*RASGRF2*	-2.53	0.01111136	*LEP*	3.25	0.0183936	*S1PR5*	-2.71	0.01573084
SMOX	2.64	0.00017096	*PRDM9*	-2.58	0.04165992	*PRR23A*	3.23	0.01742533	*KLK13*	-2.71	0.01390964
CPNE6	2.57	0.04621476	*CCDC114*	-2.63	0.03379949	*QKI*	3.22	0.00353718	*TNFSF18*	-2.81	0.00487245
SEMA6B	2.55	0.00260665	*ADAM12*	-2.70	0.03898422	*SULT1A2*	3.22	0.00099811	*SERPIND1*	-3.00	0.01405119

Table 1. Top Serotonin- and 5-HT$_{2B}$ dependent genes

5HT-dependent gene expression in macrophages is mainly mediated by the 5-HT7 - cAMP-PKA signaling axis.- To dissect the process of gene upregulation in response to 5HT, kinetic analysis was performed by determining the changes in gene expression after 2, 4, 6 and 12 hours of exposure to 5HT. Surprisingly, *EREG, IL1B*, and *TREM1* gene expression was significantly upregulated after 2 hours of exposure to 5HT (*Figure 2*), whereas those of *PDE2A, PDE7B, THBS1* and *MET* were only observed after 4 and 6 hours, and only PDE2A expression remained significantly elevated after 12 hours (*Figure 2*). The heterogeneity of responsiveness to 5HT suggested the existence of different pathways that mediate the 5HT-dependent upregulation of gene expression in macrophages. As we have previously demonstrated that 5HT alters the expression of macrophage polarization markers via engagement of 5-HT_{2B} and 5-HT_7 receptors (20), we hypothesized that 5-HT_7 might mediate most of the observed 5HT-induced gene expression changes. To address this issue, macrophages were treated with a 5-HT_7 specific agonist (LP12), or exposed to 5HT in the presence of a 5-HT7 antagonist (SB269970). The presence of the 5-HT_7 antagonist SB269970 abrogated the 5HT-triggered upregulation of *EREG, PDE2A, PDE7B* and *TREM1* (*Figure 3A*). Moreover, these genes, together with *THBS1* and *MET*, were significantly upregulated upon exposure of macrophages to the 5-HT_7 agonist LP12 (*Figure 3B*). In the same line, 6 hours LP-12-treatment lead to a reduction LPS-induced TNFα and IL-12p40 production, whereas it had no effect on IL-10 levels (*Figure 3C*). Therefore, these experiments allowed us to conclude that a significant proportion of the 5HT-dependent gene expression changes in human macrophages are dependent on 5-HT_7 engagement.

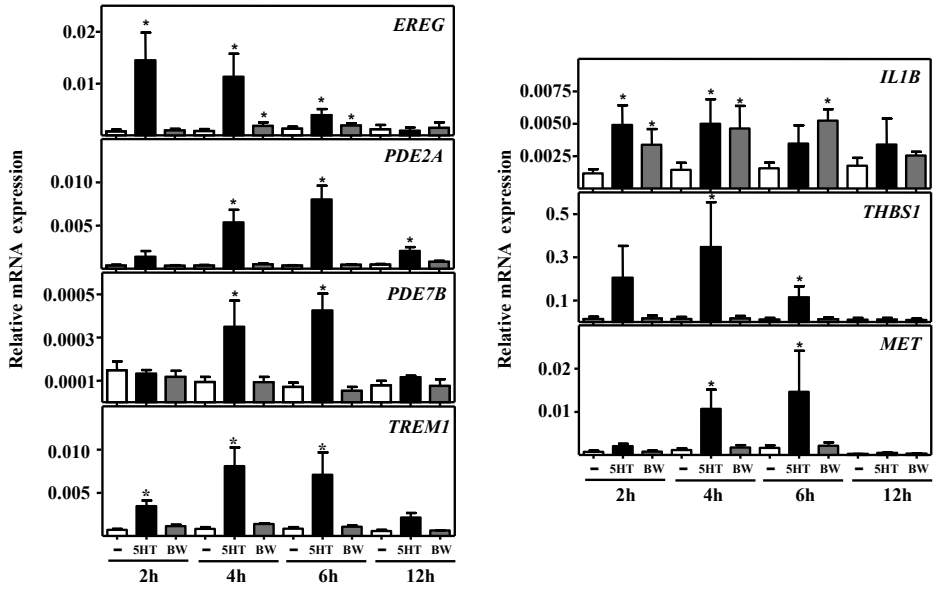

Figure 2.- Influence of 5HT and the 5HT2B agonist BW723C86 on the gene expression profile of human macrophages.- Relative expression of the indicated genes in macrophages either untreated (-) or treated with 5HT or the 5-HT2B agonist BW723C86 (BW) for 2, 4, 6 and 12 hours. Results are expressed as Relative Expression, which indicates the expression of the corresponding mRNA relative to the GAPDH mRNA levels in each sample. Three independent experiments were performed and mean and standard deviations are shown. (*, $p<0.05$).

Upon ligand binding, 5-HT_7 augments cAMP (21), whose targets include PKA and exchange factor directly activated by cAMP (Epac) (22). Since a number of 5HT-regulated genes were related to the cAMP signalling

pathway (*Supplementary Figures 1 and 2*), and were directly induced by a 5-HT_7 receptor agonist (*Figure 3B*), we assessed the ability of cAMP analogs to upregulate the expression of 5HT-regulated genes. Br-cAMP and dBr-cAMP significantly upregulated the expression of genes significantly altered by the 5-HT_7 receptor agonist (*Figure 4A, B*). Furthermore, all of them were significantly upregulated by either forskolin or the PKA-specific activator 6-Bnz-cAMP, but not by the specific Epac activator cAMP analog 8-(4-chlorophenylthio)-2′-O-methyl-cAMP (8-pCPT) (*Figure 4A, B*).

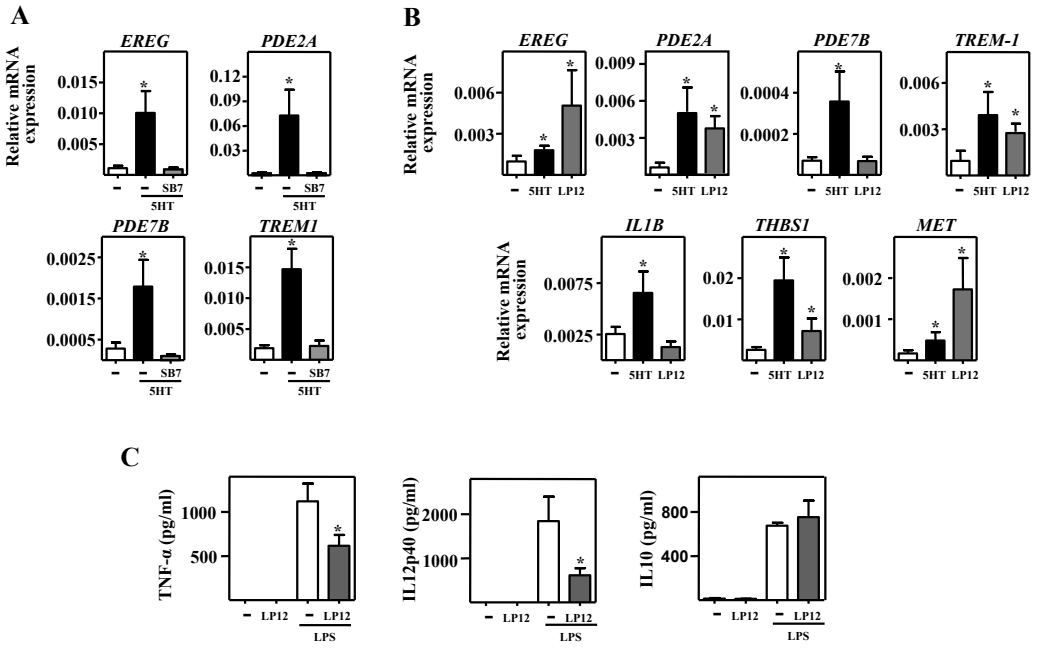

Figure 3.- Influence of the 5HT7 receptor on the gene expression profile of human macrophages.- A. Relative expression of the indicated genes in macrophages either untreated (-) or treated with 5HT (6 h) in the absence (-) or the presence of an antagonist of the 5-HT7 receptor. Results are expressed as Relative Expression, which indicates the expression of the corresponding mRNA relative to the GAPDH mRNA levels in each sample. Three independent experiments were performed and mean and standard deviations are shown. (*, $p<0.05$). **B.** Relative expression of the indicated genes in macrophages either untreated (-) or treated (6 h) with 5HT or the -HT7 receptor agonist LP12. Results are expressed as Relative Expression, that indicates the expression of the corresponding mRNA relative to the GAPDH mRNA levels in each sample. Three independent experiments were performed and mean and standard deviations are shown. (*, $p<0.05$). **C.** Determination of TNFα, IL-12p40 and IL-10 levels in culture supernatants of non-treated (-) or LP12-treated (6 h) macrophages, after stimulation with LPS (10 ng/ml) for 18 hours. Each determination was performed in triplicate on three independent macrophage samples, and means and standard deviations are shown (n=4, *, $p < 0.05$ compared to the cytokine levels in LPS-treated macrophages (LPS))

To definitively establish the involvement of the 5-HT_7-cAMP-PKA axis in the 5HT-dependent upregulation of the aforementioned genes, macrophages were exposed to the 5-HT_7 agonist LP12 in the presence of the PKA inhibitor RP-8-CPT-cAMPS. As expected, the upregulation of *EREG, PDE2A, TREM1, THBS1* and *MET* by LP12 was significantly reduced in the presence of the PKA inhibitor RP-8-CPT-cAMPS (*Figure 4A, B*). Therefore, the 5HT-initiated upregulation of *PDE2A, PDE7B, EREG, TREM1, THBS1* and MET is mediated by 5-HT7 and the subsequent cAMP-dependent activation of PKA, without a significant contribution of Epac.

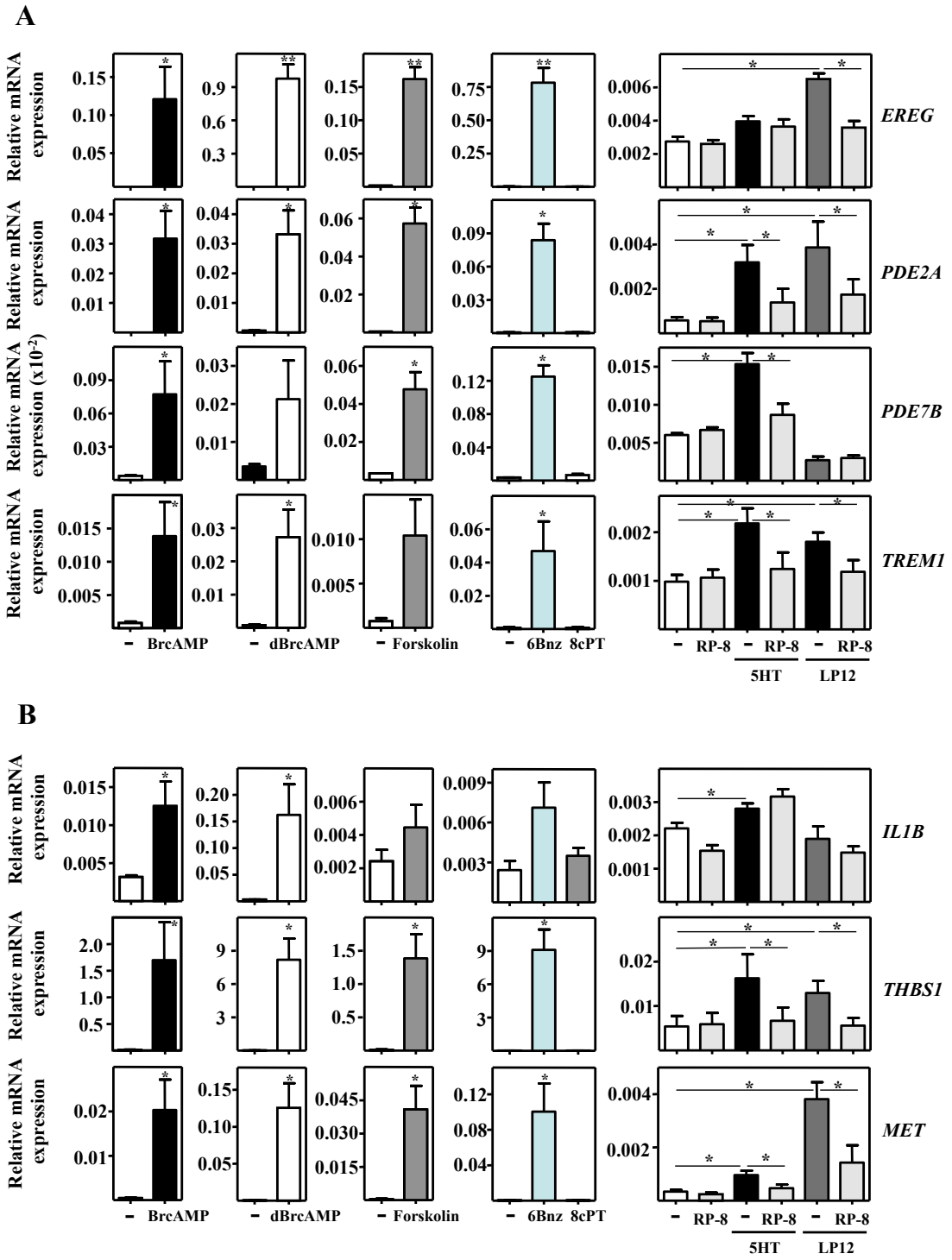

Figure 4.- Influence of the cAMP-dependent signaling pathways on the gene expression profile of human macrophages.- A,B. Relative expression of the indicated genes in macrophages either untreated (-) or treated (6 h) with Br-cAMP, dBr-cAMP, Forskolin, 6-Bnz-cAMP (6Bnz) or 8-(4-chlorophenylthio)-2′-O-methyl-cAMP (8cPT). Right panels: Relative expression of the indicated genes in macrophages either untreated (-) or treated (6 h) with 5HT or the 5-HT7 receptor agonist LP12 and either in the absence or the presence of the PKA inhibitor RP-8-CPT-cAMPS (RP-8). In all cases, results are expressed as Relative Expression, that indicates the expression of the corresponding mRNA relative to the GAPDH mRNA levels in each sample. Three independent experiments were performed and mean and standard deviations are shown. (*, $p<0.05$; **, $p<0.01$).

The functional relevance of the 5HT-dependent gene expression changes was corroborated through evaluation of changes at the protein level. To that end, the release of IL1β and Epiregulin, the proteins encoded by *IL1B*

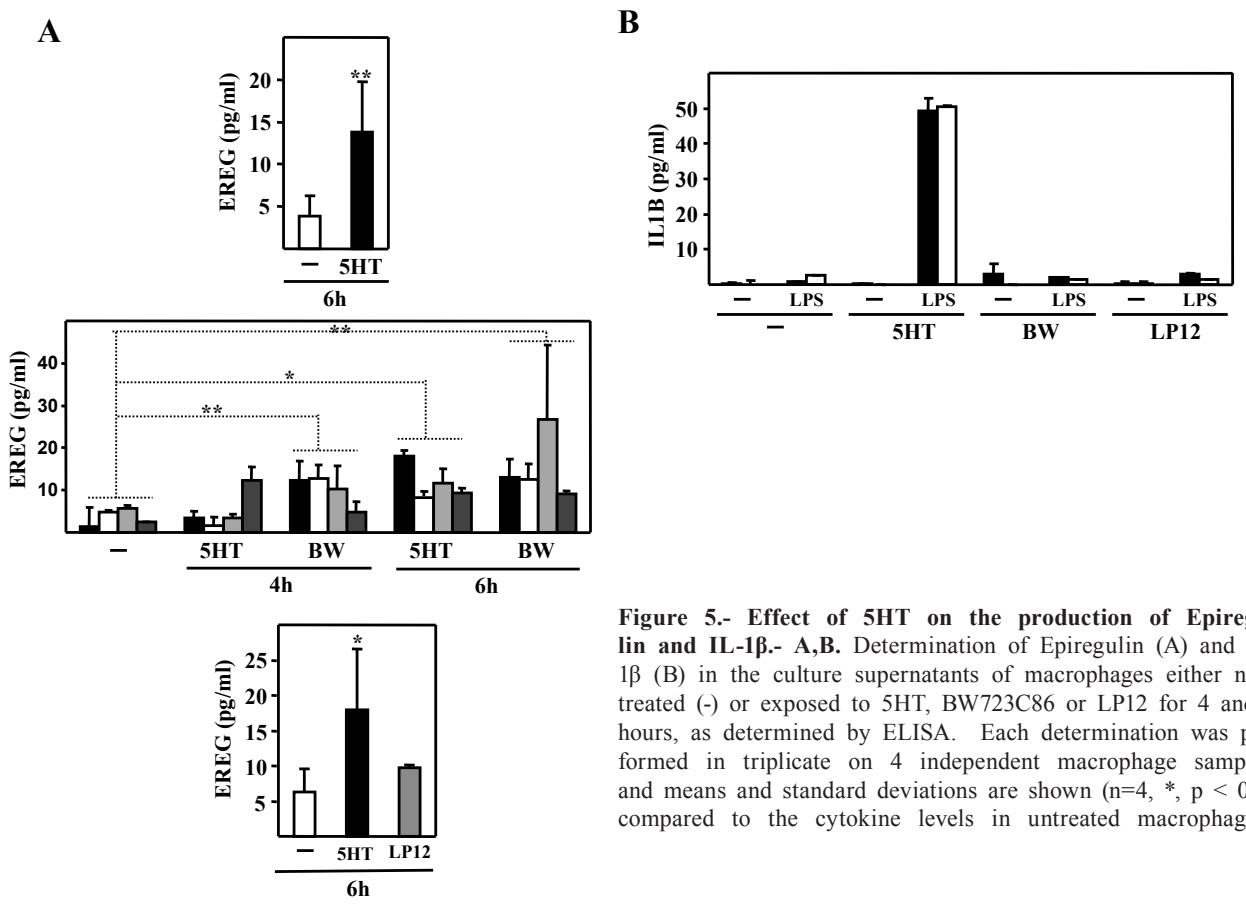

Figure 5.- Effect of 5HT on the production of Epiregulin and IL-1β.- A,B. Determination of Epiregulin (A) and IL-1β (B) in the culture supernatants of macrophages either non-treated (-) or exposed to 5HT, BW723C86 or LP12 for 4 and 6 hours, as determined by ELISA. Each determination was performed in triplicate on 4 independent macrophage samples, and means and standard deviations are shown (n=4, *, $p < 0.05$ compared to the cytokine levels in untreated macrophages).

and *EREG* respectively, was measured in macrophages exposed to 5HT. As shown in *Figure 5*, epiregulin was detected at detectable levels in macrophages treated with 5HT or even the 5-HT_{2B} agonist (*Figure 5A*). Conversely, no IL1β was found in the supernatant of 5HT-treated macrophages (*Figure 5B*).

The 5-HT2B receptor also contributes to the serotonin-dependent gene expression in macrophages.- We have previously shown that the 5-HT_{2B} receptor constitutes a marker for M2 macrophage polarization in vitro and in vivo, and that 5-HT_{2B} influences macrophage polarization (20). As a mean to determine whether 5-HT_{2B} also contributed to the 5HT-dependent gene expression profile in macrophages, transcriptional profiling was done on macrophages that had been exposed to the 5-HT_{2B} agonist BW723C86 for 6 hours (*Figure 6A*). More than 400 genes were regulated upon exposure to BW723C86 (*Figure 6B*). Microarray data were verified on independent macrophage samples, and BW723C86 was found to regulate the expression of *IL1B, IFIT1, IFIT2, RSAD2, CXCL10, ETV7* and *TNFSF18* (*Figures 2* and *6C*). Except for the case of *IL1B* and *TNFSF18,* none of these genes were significantly affected by 5HT (*Figure 2* and *6C*), thus suggesting a predominance of 5-HT_7 receptor in the 5HT-dependent gene profile in human macrophages. In fact, comparison of the 5HT- and

BW723C86-dependent profiles revealed that only a gene subset was regulated by both agents (*Figure 6D* and *Table I*). Altogether, these results illustrate that 5HT modifies the macrophage gene expression through more than one receptor (at least 5-HT_7 and 5-HT_{2B}), an explanation that is compatible with our previous report on the modulation of macrophage polarization by both receptors (20).

Identification of a 5-HT_{2B}-mediated link between 5HT and IFN signaling.- Gene ontology analysis indicated that BW723C86 affects the expression of genes associated with Coagulation, Interferon Signaling, Aryl Hydrocarbon Receptor Signaling, Chemokine Signaling and EIF2 Signaling (*Supplementary Figure 1*), whereas Ingenuity Pathway Analysis revealed a significant link between BW723C86-upregulated genes and the IFN type I signaling pathway (*Supplementary Figure 2*). Besides, SOCS1 ($p=6.04 \times 10^{-6}$) and Interferon ($p=2.07 \times 10^{-5}$) were predicted as Upstream Regulators of BW723C86-regulated genes (*Supplementary Figure 2*). Therefore, we searched for interferon (IFN)-regulated genes within the 5-HT_{2B} receptor-regulated genes. Interestingly, 16% of the 177 genes upregulated by BW723C86, but not 5HT, turned out to be regulated by IFN (http://www.interferome.org). Along the same line, 18% of the genes upregulated by either BW723C86 or 5HT have been previously identified as IFN-responsive (http://www.interferome.org). Importantly, the specific involvement of 5-HT_{2B} receptor in the gene changes observed upon BW723C86 treatment was demonstrated through the use of the 5-HT_{2B} receptor antagonist SB204741, which inhibited the BW723C86-mediated

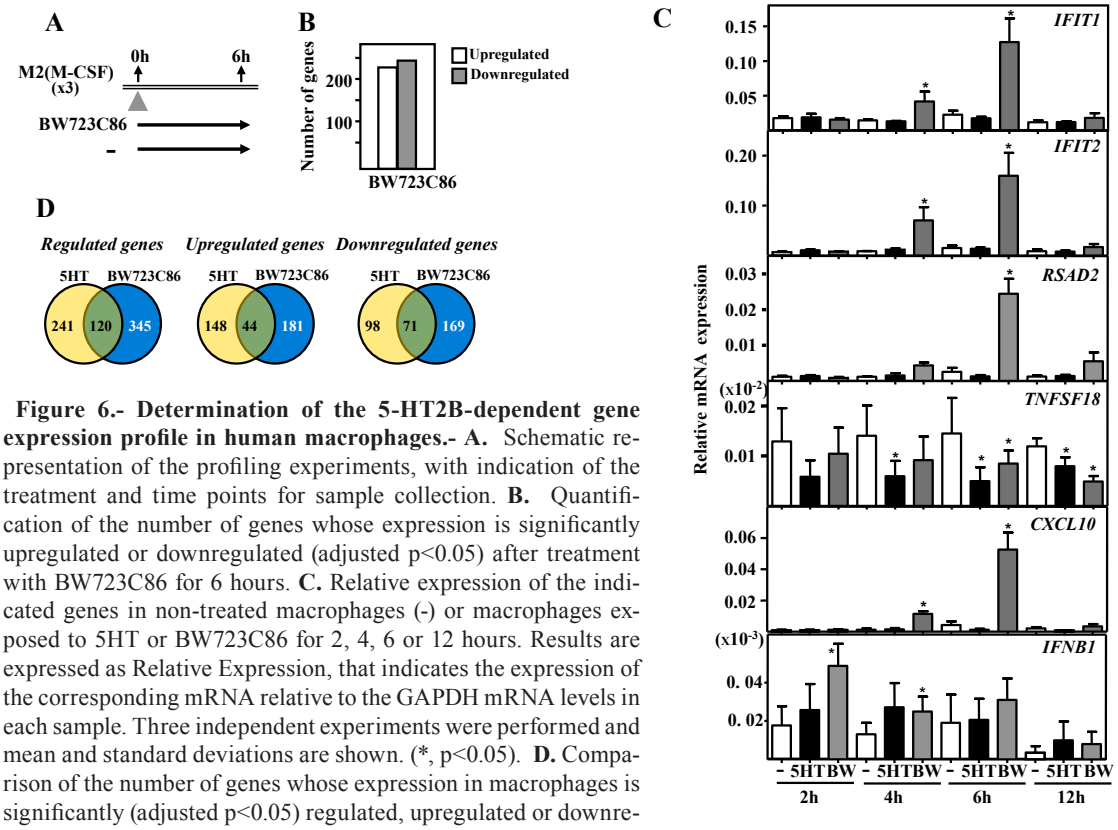

Figure 6.- Determination of the 5-HT2B-dependent gene expression profile in human macrophages.- A. Schematic representation of the profiling experiments, with indication of the treatment and time points for sample collection. **B.** Quantification of the number of genes whose expression is significantly upregulated or downregulated (adjusted $p<0.05$) after treatment with BW723C86 for 6 hours. **C.** Relative expression of the indicated genes in non-treated macrophages (-) or macrophages exposed to 5HT or BW723C86 for 2, 4, 6 or 12 hours. Results are expressed as Relative Expression, that indicates the expression of the corresponding mRNA relative to the GAPDH mRNA levels in each sample. Three independent experiments were performed and mean and standard deviations are shown. (*, $p<0.05$). **D.** Comparison of the number of genes whose expression in macrophages is significantly (adjusted $p<0.05$) regulated, upregulated or downregulated after treatment (6 hours) with either 5HT or BW723C86.

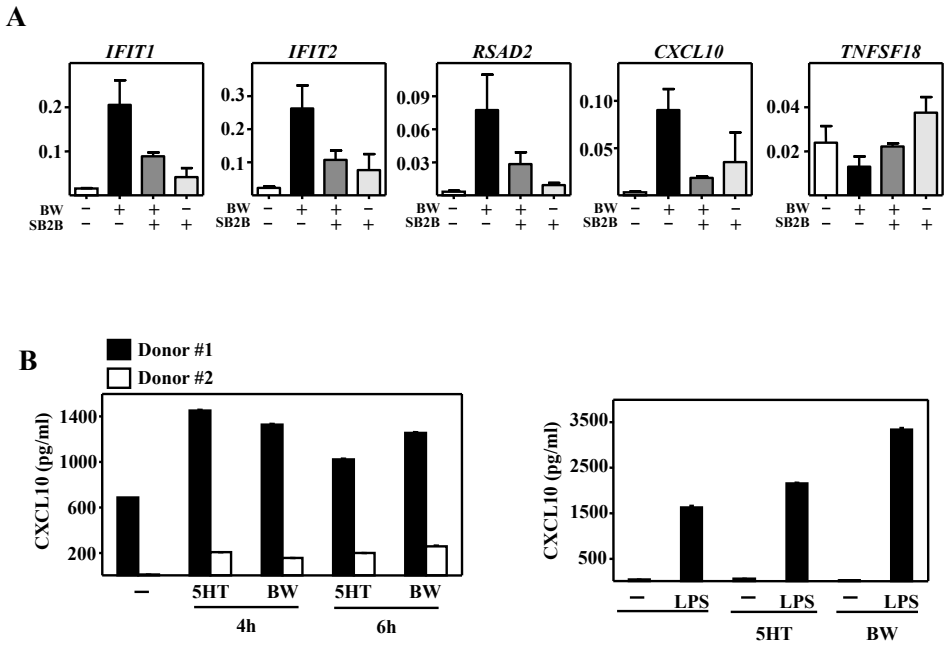

Figure 7.- The 5-HT2B receptor mediates the gene expression changes induced by BW723C86 on macrophages.- A. Relative expression of the indicated genes in non-treated macrophages (-), macrophages exposed for 6 hours to BW723C86 in the presence or in the absence or the 5-HT2B antagonist SB SB204741 (SB2B), or macrophages exposed solely to the 5-HT2B antagonist. Results are expressed as Relative Expression, that indicates the expression of the corresponding mRNA relative to the GAPDH mRNA levels in each sample. Three independent experiments were performed and mean and standard deviations are shown. (*, $p<0.05$). **B.** Determination of CXCL10 levels in culture supernatants of non-treated (-) or 5-HT-treated or BW723C86 (6 h) macrophages, after stimulation with LPS (10 ng/ml) for 18 hours. Each determination was performed in triplicate on two independent macrophage samples, and means and standard deviations are shown (n=2).

regulation of *IFIT1, IFIT2, CXCL10, RSAD2* and *TNFSF18* genes (*Figure 7A*). These findings support the existence of a potential link between 5HT and interferon signaling. Moreover, the ability of BW723C86 to upregulate the expression of paradigmatic IFN-regulated genes like *IFIT1, IFIT2, RSAD2, OASL, CXCL10* and *ETV7* (*Table I* and *Figure 6C*) and basal levels of *CXCL10* protein (*Figure 7B*) suggests that such a link is primarily 5-HT_{2B}-dependent.

DISCUSSION

We have previously reported that 5HT skews macrophage polarization (20). The data in the present manuscript further extends this finding, as we have determined the whole range of polarization-associated markers specifically affected by 5HT. Comparison of the 5HT- and BW723C86-dependent gene expression profiles with those of proinflammatory M1 and anti-inflammatory M2 macrophages (23) indicates that 5HT modulates a higher number of M2-specific genes (including *MS4A7, TTYH2, ISG20, RGS2, OLFML2B, HMOX1, S1PR1* and *NAMPT)* (*Supplementary Figure 3*). The ability of 5HT to alter the expression of *HMOX1* (that encodes

Heme-Oxygenase 1) and *S1PR1* (that codes for the sphingosine-1-phosphate (S1P) receptor 1) is of special interest because the *S1PR1* agonist FTY720 increases the expression of Heme-Oxygenase 1, that exhibits anti-apoptotic and anti-inflammatory activity, and because Heme-Oxygenase 1 favors the acquisition of an anti-inflammatory phenotype in macrophages exposed to S1P-containing apoptotic cell supernatants (24,25). Therefore, it is conceivable that 5HT participates in the M1-to-M2 macrophage polarization switch that takes place in the presence of apoptotic cells and apoptotic cell-derived products.

In peripheral tissues, 5HT regulates cell proliferation, inflammation and tissue repair (6,26,27). 5-HT_7 is primarily expressed in smooth muscle cells of blood vessels (28) and the gastrointestinal tract (29), and also detected in kidney, liver, pancreas and spleen. Interestingly, the physiological processes regulated by 5HT (cell proliferation, tissue repair, inflammation) are also critically modulated by tissue macrophages in a polarization-dependent manner (30). However, and in spite of its potential *in vivo* significance, the role of 5HT on innate immune cells is not yet completely understood (8). Within the immune system, 5-HT_7 has been found to be expressed by naive T cells (13), monocytes (31) and dendritic cells (32-35). We previously showed that the modulation of the phenotypic and functional macrophage polarization by 5-HT is mediated by both 5-HT_{2B} and 5-HT_7 receptors (20). In the present report we describe the identification of the 5HT-dependent gene expression profile on human macrophages, and demonstrate that most transcriptional effects of 5HT on macrophages are mediated by the 5-HT_7-PKA signaling axis because 5-HT_7 agonists and antagonists reproduce or neutralize the majority of 5HT-mediated gene expression changes. In addition, we provide evidences for the involvement of 5-HT_{2B} on the 5HT effects on human macrophages, and illustrate the existence of a link between 5-HT_{2B}-initiated signals and the type I IFN signaling pathway. The identity of the genes specifically regulated after 5HT binding to 5-HT_7 and/or 5-HT_{2B} sheds new light on the ability of 5HT to modulate cell proliferation (by promoting the expression of growth factors and growth factor receptors) and to influence macrophage effector functions (by modulating the expression of genes that control macrophage activation). Thus, our results place 5-HT_7 as a potentially relevant molecule for modulation of macrophage effector functions under physiological and pathological settings.

In the case of the central nervous system, 5-HT_7 mRNA has found in thalamus, hypothalamus, cerebral cortex, hippocampus and amygdala (36-38). In line with the control of various cerebral functions by 5HT, 5-HT_7 regulates circadian rhythms, thermoregulation, learning and memory (2,3,38-42), affects depression and activation of rapid-eye-movement (REM) sleep (2-4), and is a potential therapeutic target for pain and migraine,

schizophrenia, anxiety, cognitive disturbances, and inflammation (2). Our results on human macrophages are in line with these previous findings, because the signaling ability of 5-HT_7 is compatible with the receptor exerting a modulatory action on the process of macrophage activation. In fact, given the expression of 5-HT_7 in microglia cells (43), it is tempting to speculate that 5-HT_7-expressing microglia could participate in the above mentioned physio-pathological processes. This hypothesis would be also compatible with recent evidences for the implication of alternatively IL-4-activated macrophages in maintaining adaptive thermo-genesis (44) and improving learning and memory (45-47), two processes that are directly controlled by 5-HT_7.

The predicted upstream regulators of 5-HT_7–regulated genes that we have identified in human macrophages also fit well with 5-HT_7-initiated signalling pathways in other cell types. 5-HT_7 stimulation affects gene expression via activation of ERK1/2, PKA (48), Akt (49), p38 MAPK and protein kinase Cε (PKCε) (50), and stimulation of Cdc42 and RhoA GTPases (51). 5-HT_7 couples positively to adenylate cyclase (AC) through activating Gαs, resulting in increased of cAMP levels and activation of PKA and Epac1/2 (22). In fact, the activation of Cdc42 and RhoA occurs via 5-HT_7-Gα12 coupling and regulates the transcription of serum response factor (SRF)-regulated genes (51). Probably in a PKA- (48,52) or Epac-dependent manner (53), 5-HT_7 engagement also triggers ERK1/2 activation in naive T cells (13,54), and monocytes (31), where it also prevents apoptosis (31). In addition, 5-HT_7 activates NF-κB in naive T cells, monocytes and dendritic cells (13,31,35). Our results indicate the predominance of the 5-HT_7-AC-PKA pathway in human macrophages exposed to 5HT, as most 5HT-regulated genes could be induced by PKA activators and their 5HT-responsiveness is blocked by PKA inhibitors. Furthermore, this result would indicate that, like in other cell types (51) (and our unpublished results), 5HT would activate CREB in macrophages. If so, considering that CREB activation favours the acquisition of an M2 polarization state (55), the 5HT-dependent macrophage polarization shift towards M2 that we have previously reported (20) would be dependent on such a 5-HT_7-AC-PKA-CREB signaling axis. In addition, and taking into account the importance of cAMP-initiated signaling for limiting the effector functions of pro-inflammatory stimuli (56,57), the 5-HT_7-AC-PKA-CREB pathway could play a role in inflammation resolution in cases where platelet de-granulation takes place. This issue deserves additional investigation because we have observed that 5HT also increases mRNA levels for various phosphodiesterases, which limit cAMP availability.

Regarding target genes, 5-HT_7 ligation augments pro-inflammatory cytokine production (IL-12p40, IL-1β, and IL-6) in CD11c+ dendritic cells, probably via NF-kB activation (35), enhances IL-6 synthesis in astrocytoma

and human microglia cells (43,50), and increases cAMP and secretion of IL-1β, IL-6, IL-12p40 and CXCL8, but lowers LPS-induced TNF-α release, in monocytes (11). Since 5-HT_7-induced increased IL-6 synthesis peaks at 4 and 24 h after 5HT stimulation, the existence of two independent 5-HT_7-initiated signal transduction pathways has been hypothesized. However, it is also possible that 5HT might trigger the production of a factor promoting IL-6 release (50). Our results suggest that the 5-HT_7-AC-PKA-CREB signaling pathway might contribute to the latter mechanism, as it mediates the positive effect of 5HT onthe gene (*IL1B*) encoding IL-1β, a potent inducer of IL-6 synthesis.

Although to a lower extent than 5-HT_7, our results indicate that 5HT also modulates macrophage gene expression via 5-HT_{2B} (*Figure 8*). This is specially relevant from a pathologic point of view, because 5-HT_{2B} mediates the deleterious effect of 5HT in diseases like pulmonary arterial hypertension (PAH) and fibrosis (15,58), liver fibrosis (59) and cardiac hypertrophy (60). Besides, 5-HT_{2B} controls bone mass via osteoblast proliferation (61), and modulates hepatocyte proliferation in chronic liver disease (62). The pathological link between 5-HT_{2B} and cell proliferation/fibrosis is supported by its ability to stimulate the production of extra-cellular matrix proteins (63) and, in fact, targeting 5-HT_{2B} has been proposed as an effective antiproliferative and antifibrotic strategy (64). Our gene expression profiling experiments have identified growth factors that might potentially mediate the cell growth-promoting action of 5-HT_{2B}, as exposure of macrophages to either 5HT or an 5-HT_{2B} agonist lead to enhanced expression of the *EREG* gene-encoded epiregulin growth factor. Epiregulin is a member of the epidermal growth factor family which binds to the epidermal growth factor receptor (EGFR) and most members of the ERBB family. Epiregulin is a growth factor involved in cancer development whose expression is regulated by cAMP (65). Importantly, epiregulin functions as a autocrine/paracrine factor for vascular smooth muscle cells (VSMC)(66), and contributes to vascular remodeling (67). Therefore, epiregulin might constitute a link between 5-HT_{2B} on macrophages and the abnormal neomuscularization of small pulmonary arteries, adventitial proliferation and vascular remodelling that occurs in pulmonary arterial hypertension, whose development is dependent on bone marrow-derived cells (15).

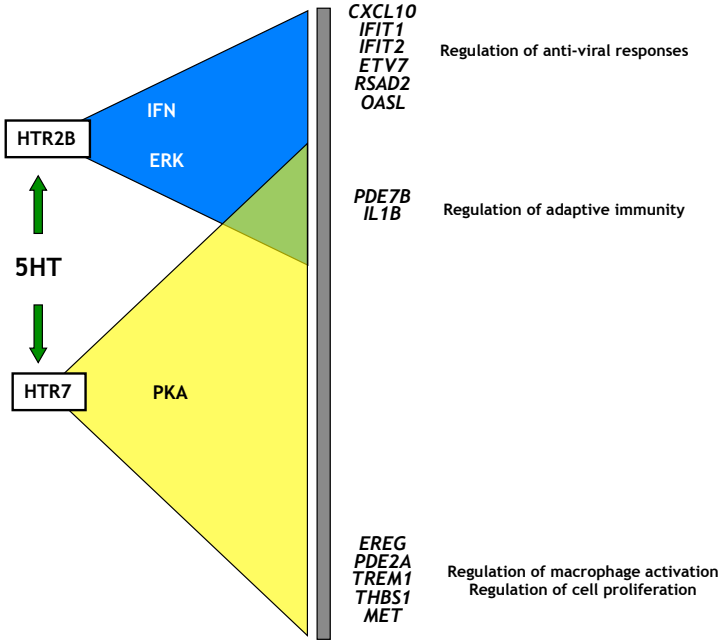

Figure 8.- Schematic representation of the gene expression profiles of macrophages that are regulated by either 5HT or the 5-HT$_{2B}$ agonist BW723C86.

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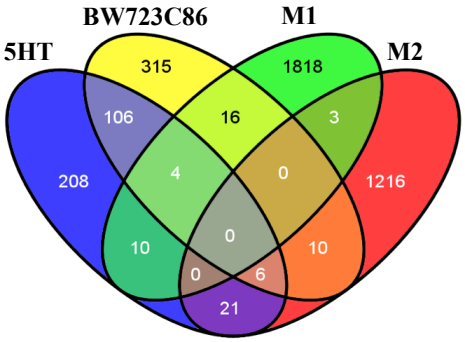
Ingenuity Canonical Pathways	-log(p-value)	Ratio	Molecules
5HT-regulated genes			
cAMP-mediated signaling	3.12E00	5.02E-02	*GRK4,PDE2A,RGS2, PDE7B,PKIB,CREM,PDE4A,S1PR1,FPR2,HTR1D,PKIG*
VDR/RXR Activation	2.77E00	7.41E-02	*IFNG, WT1,FOXO1,RUNX2,THBD,HSD17B2*
Docosahexaenoic Acid (DHA) Signaling	2.44E00	8.16E-02	*ALOX15,FOXO1,IL1B,PNPLA2*
Clathrin-mediated Endocytosis Signaling	1.97E00	4.1E-02	*VEGFA,MET,CDC42,GAK,FGF8,FIGF,ITGB8,MYO1E*
mTOR Signaling	1.9E00	3.83E-02	*VEGFA,HMOX1,DDIT4,FIGF,RPS6,RPS6KA5,EIF3C/EIF3CL,RPS23*
BW723C86-regulated genes			
Interferon Signaling	2.41E00	1.11E-01	*IFIT3,IFIT1,IFNG ,IFNA21*
Coagulation System	2.36E00	1.05E-01	*VWF,F7,PLAT,SERPIND1*
Aryl Hydrocarbon Receptor Signaling	2.31E00	5.03E-02	*RB1,NQO1,MAPK8,TGFB3,IL1B,NF1B,ALDH3A1,CYP1B1*
Atherosclerosis Signaling	2.12E00	5.34E-02	*COL1A2,ITGB2,PLA2G6,IFNG , CXCR4,IL1B,COL11A2*
Chemokine Signaling	1.99E00	6.85E-02	*CALML5,CXCR4,MAPK8,PPP1R12B,CAMK2G*
MSP-RON Signaling Pathway	1.94E00	7.84E-02	*ITGB2,IFNG, ACTB,NOS2*
EIF2 Signaling	1.57E00	3.96E-02	*RPL21,RPL23A,RPS21,RPS3,RPS23,RPS27A,RPS12,RPL37*

Supplementary Figure 1.- Identification of the canonical signaling pathways that potentially control the expression of 5HT- and BW723C86-regulated genes, as determined by the Ingenuity Pathway Analysis, with indication of its statistical significance (adjusted p-value, p) and the molecules associated.

	Upstream Regulator	p-value
5HT upregulated genes	LY294002	2.24E-07
	beta-estradiol	6.28E-07
	Tgf beta	2.06E-06
	forskolin	3.17E-06
	lipopolysaccharide	4.26E-06
	HGF	6.43E-06
	prostaglandin E2	1.78E-05
	IL6	2.05E-05
	IL1B	4.15E-05
	SMAD7	7.04E-05
BW723C86 upregulated genes	TNF	2.07E-08
	IFNA2	5.09E-07
	SOCS1	1.73E-06
	lipopolysaccharide	4.26E-06
	IFNG	1.01E-05
	IFNA1/IFNA13	1.15E-05
	Interferon alpha	1.28E-05
	IFN TYPE 1	4.19E-05
	BACH1	5.78E-05
	IFN Beta	6.14E-05
5HT & BW723C86 Upregulated genes	IL33	1.10E-04
	simvastatin	3.55E-04
	8-bromo-cAMP	3.72E-04
	CD200	4.73E-04
	NR1H3	4.87E-04
	thiazolidinedione	5.40E-04
	C5	9.43E-04
	IFN Beta	1.01E-03
	SMAD7	1.56E-03

Supplementary Figure 2.- Identification of the potential upstream regulators of the expression of 5HT- and BW723C86-upregulated genes, as determined by the Ingenuity Pathway Analysis, with indication of its statistical significance (adjusted p-value, p).



Supplementary Figure 3.- Quantification of the 5HT- and BW723C86-regulated genes whose expression significantly differs in M1(GM-CSF) (M1) and M2(M-CSF) (M2) macrophages (> 2-fold; adjusted p<0.05).

Intravenous immunoglobulin promotes anti-tumor responses by modulating macrophage polarization

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Running title: Macrophage-dependent inhibition of tumor progression by IVIg

ABSTRACT

Intravenous immunoglobulins (IVIg) therapy is widely used as an immunomodulatory strategy in inflammatory pathologies, and is suggested to promote cancer regression. Since progression of tumors depends on their ability to re-direct the polarization state of tumor-associated macrophages (from M1/immunogenic/pro-inflammatory to M2/anti-inflammatory), we have evaluated whether IVIg limits tumor progression and dissemination through modulation of macrophage polarization. *In vitro*, IVIg inhibited proinflammatory cytokine production from M1 macrophages, and induced a M2-to-M1 polarization switch on human and murine M2 macrophages. *In vivo*, IVIg modified the polarization of tumor-associated myeloid cells in an Fcεr1γ-dependent manner, modulated cytokine blood levels in tumor-bearing animals, and impaired tumor progression via CD16 and FCεR1γ engagement, the latter two effects being macrophage-mediated. Therefore, IVIg immunomodulatory activity is dependent on the polarization state of the responding macrophages, and its ability to trigger a M2-to-M1 macrophage polarization switch might be therapeutically useful in cancer, where pro-inflammatory or immunogenic functions should be promoted.

INTRODUCTION

Macrophages exhibit a huge functional plasticity and, in response to endogenous and non-self stimuli, can acquire a continuum of polarization states (1-4). Microbe-derived factors, or cytokines like IFNγ, GM-CSF or TNFα, promote in macrophages the acquisition of pro-inflammatory, bactericidal, tumor suppressive and immunogenic activities, a process commonly referred to as "classic" or M1 polarization and whose hallmark is the ability to release large amounts of IL-12/IL-23 (2). Conversely, cytokines like IL-4, IL-10, TGFβ or M-CSF, promote anti-inflammatory, scavenging, tumor-promoting, tissue repair and pro-angiogenic functions, all of which are grouped under the terms "alternative" or M2 polarization that endows them with the ability to produce high levels of IL-10 (3-5). M1-polarized macrophages predominate at the initial stages of an inflammatory response, whereas M2-type macrophages drive resolution of inflammation, tissue repair after injury, and maintain tissue homeostasis (6). *In vivo*, the misbalance of macrophage polarization states underlies numerous pathophysiological processes, including tumor development, autoimmune diseases and chronic inflammatory pathologies (6-8).

The switch between M1 and M2 polarization states is especially relevant in tumor initiation, progression and dissemination, which are extremely reliant on the presence and polarization state of macrophages within the tumor stroma (Tumor-Associated Macrophages, TAM) (5). The contribution of macrophages to tumor development is inferred from the poor outcome associated with enhanced levels of M-CSF, the reduced metastasis observed in $Csf1^{op/op}$ mice (9, 10), and the positive correlation between high content of TAM and a bad prognosis (11). Depending on their polarization status, macrophages can either promote antitumor immune responses or contribute to tumor progression (12). In fact, as tumor progresses, TAM develop an immunosuppressive and pro-tumoral phenotype which fuels tumor growth, metastasis and suppression of tumor-specific immune responses (13).

Intravenous immunoglobulin (IVIg) is a preparation of polyclonal and poly-specific immunoglobulins derived from the plasma of thousands of healthy donors. IVIg therapy is FDA-approved for primary immunodeficiencies, Immune Thrombocytopenic Purpura and Kawasaki´s disease, and is beneficial for multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus (14, 15). Previous reports have evidenced that IVIg exerts potent immunomodulatory actions in immunodeficiency syndromes, autoimmune diseases and infectious processes (16). The molecular and cellular basis for the IVIg immunomodulatory action, including the identity of the biologically active constituents in IVIg and its specific cell surface receptors and targets cells, remains to be completely clarified (17-20). In the present report we demonstrate that IVIg promotes an M2-to-M1 macrophage polarization switch through ligation of activating Fc receptors in both human and mouse macrophages, and that *Fcgr3* and *Fcer1g* mediate the IVIg-induced re-polarization of tumor-associated myeloid cells and inhibition of tumor progression and metastasis *in vivo*.

MATERIALS AND METHODS

Macrophage differentiation, cell culture and treatments.- Human peripheral blood mononuclear cells (PBMC) isolation was carried out as described previously (21). Human TAM were obtained from the pleural fluid of a metastatic breast adenocarcinoma patient, after obtaining written informed consent and following Medical Ethics committee procedures (Hospital General Universitario Gregorio Marañón), and using CD14 microbeads as described (22). Human postnatal thymocytes were isolated from thymus fragments removed during corrective cardiac surgery of patients aged 1 month- 4 years, after providing informed consent in accordance with the Declaration of Helsinki. Thymocyte cell suspensions were enriched in non-T cells by

sheep erythrocyte rosetting as previously described (23). Intrathymic macrophages (>95% CD13+ CD11c+ CD14+) were obtained from the resulting cell fraction by positive selection using a PE-labeled anti-CD14 monoclonal antibody and anti-PE microbeads (Miltenyi Biotec). Bone marrow-derived macrophages (BMDM) were obtained as described previously (24,25). For activation, macrophages were treated with E.coli 055:B5 lipopolysaccharide (LPS) (100 ng/ml for mouse macrophages, 10 ng/ml for human macrophages) for 24 hours. The B16F10 mouse melanoma cell line (C57BL/6 origin), the mouse MC38 colon carcinoma cancer line, and the highly invasive human BLM melanoma cell line (kindly provided by Dr. J. Teixidó, CIB/CSIC, Madrid, Spain), were maintained in RPMI (MC38) or DMEM (BLM, B16F10) medium supplemented with 10% FCS, at 37ºC in a humidified atmosphere with 5% CO_2. Fully polarized macrophages were exposed to 10 mg/ml IVIg (Privigen ®, CSL Behring) for 24 hours. For Syk inhibition assays, differentiated macrophages were treated with vehicle (H_2O) or piceatannol (100 μM, Calbiochem) 1 hour before IVIg treatment. To determine the CD16 contribution, an anti-human CD16 F(ab)'2 monoclonal antibody (LSBio, clone 3G8), or an isotype-control mouse IgG1 F(ab)'2, was used at 20 μg/ml before IVIg treatment.

Quantitative real-time RT-PCR.- Total RNA was extracted using the RNeasy® Mini kit or AllPrep® DNA/RNA/Protein Mini kit (Qiagen) following manufacturer's guidelines. cDNA was synthesized using the Reverse Transcription System kit (Applied Biosystems). Oligonucleotides for selected genes were designed according to the Roche software (Universal Probe Roche library). Quantitative real-time PCR (qRT-PCR) was performed using custom-made panels (Roche Diagnostics) or standard plates on a LightCycler® 480 (Roche Diagnostics) or a iQTM5 (Biorad), respectively. An extensive battery of genes differentially expressed between M1 and M2 macrophages were included in our assays (a total of 33 genes, out of which thirteen were previously identified as M1 marker genes and twenty as M2 marker genes) (21,26,27). Assays were made in triplicate and results normalized according to the expression level of GAPDH or to the mean of the expression level of endogenous reference genes HPRT, SDHA and TBP. Results were expressed using the ΔΔCT method for quantitation.

ELISA.- Culture supernatants from LPS-treated (24h) human macrophages were assayed for the presence of cytokines using commercially available ELISA for TNF-α, IL-10, IL-6 (ImmunoTools GmbH), IL-12p40, CCL-2 (OptEIA™ IL-12p40 set, BD Pharmingen) and Activin A (R&D Systems). LPS-treated mouse macrophage supernatants were tested for Il-10, Tnf-α and Ccl-2 using available ELISA (BioLegend). ELISA were performed following the protocols supplied by the manufacturers.

Cell Proliferation Assays.- BLM cells were plated (5×103 cells/well), allowed to adhere for 24 h, and exposed to culture supernatants from untreated or IVIg-treated human macrophages for 48 h. Cell proliferation was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma). Complete media was used as control to determine the basal BLM cell proliferation.

Mouse tumoral models.- For the pulmonary metastasis animal model, 6-to-8-week-old wild-type C57BL/6, Fcgr3$^{-/-}$ and Fcer1g$^{-/-}$ mice (kindly provided by Dr. Jordi Ochando, Mount Sinai School of Medicine, NY, USA) were used for all experiments. Mice were injected intravenously (i.v., tail vein) with 3 x 10^5 B16F10 melanoma cells in 0.1 ml of sterile PBS on day 0, and with 400 µl of IVIg (100 mg/ml) or PBS (control) 24 h before tumor cell injection (day -1) and on days 6 and 13. Mice were sacrificed on day 18, and lung surface metastases counted under a dissecting microscope as black nodules after bleaching in Fekete´s solution. For xenograft studies, 2–3 months old BALB/c SCID or C57BL/6 mice were injected subcutaneously (lateral thoracic wall) with 2 x 10^6 BLM cells or 5 x 10^5 MC38 cells in 0.1 ml of PBS 0.1% glucose. IVIg (400 µl, 100 mg/ml) or PBS (400 µl) was injected intravenously on day -1, 7 y 14, and mice were killed 17-21 days after cell tumor inoculation. Mice were inspected daily and the tumor volume measured as width2 ×length / 2. All protocols were approved by the CIB-CSIC Ethics Committee.

Permanent middle cerebral artery occlusion (pMCAO) in mice.- The surgical procedure was a variant of that described by Chen et al. (1986) (28) and Liu et al. (1989) (29). Mice were anesthetized with isoflurane 1.5-2% in a mixture of 80% air/20% oxygen, and body temperature was maintained at physiological levels with a heating pad during the surgical procedure and anesthesia recovery. Mice were subjected to permanent focal cerebral ischemia through the distal occlusion of middle cerebral artery (MCA) by ligature of the trunk just before its bifurcation between the frontal and parietal branches with a 9-0 suture, in combination with the occlusion of the ipsilateral common carotid artery. Physiological parameters were not significantly different among the different groups studied. Following surgery, individual animals were returned to their cages with free access to water and food. All the groups were performed and quantified in a randomized fashion by investigators blinded to treatment groups. IVIg (400 µl, 100 mg/ml) or PBS (400 µl) were injected by vein tail 10 min after MCAO. 48h after pMCAO mice were killed by an overdose of sodium pentobarbital and brain was removed, cut into 1-mm thick coronal slices and stained with 2,3,5-triphenyl tetrazolium chloride (TTC; 1% in 0.1M phosphate buffer). Infarct volumes were calculated sampling each side of the coronal sections with a digital camera (Nikon Coolpix 990), and the images were analyzed using ImageJ 1.33u (National Institutes of Health).

To exclude the brain edema effects, infarct area was corrected by the ratio of the entire area of the ipsilateral hemisphere to that of the contralateral.

LPS-Induced Endotoxin Shock model mice.- Mice received i.p. injections of 350 μg of LPS per 25 g of body weight one hour after or PBS injection. LPS was dissolved prior to injection in phosphate-buffered saline at a concentration of 10 mg/ml. Injected animals were monitored for a 7 days.

Statistical analysis.- Differences between the experimental groups in in vivo experiments were analyzed by a non-paired Student t-test. In the case of CD11b+ isolated cells, qPCR data were analyzed with the REST-2009 software from Qiagen using 5000 permutations. Statistical significance of in vitro generated data was evaluated using a paired Student t-test. In all cases, $p<0.05$ was considered as statistically significant.

RESULTS

M1 and M2 macrophages are differentially affected by IVIg.- Since IVIg is used off-label for chronic inflammatory diseases (30), where M1 macrophages critically contribute to pathology, we first investigated whether IVIg modulates the effector functions of pro-inflammatory M1 macrophages. A 24-hour exposure to IVIg led to a significant reduction in the LPS-stimulated release of TNFα, IL-12p40 and CCL2 from M1 macrophages (*Fig. 1A*), without affecting their tumor cell growth inhibitory ability (*Fig. 1C, upper pane*l). IVIg did not overtly alter the gene expression profile of M1 macrophages, as most M1 polarization-specific markers were only weakly modulated in response to the treatment (*Fig. 1D, upper panel*). The functional modulation induced by IVIg on M1 macrophages is, therefore, compatible with the previously reported anti-inflammatory activity of IVIg, and might help explaining the clinical improvement of chronic inflammatory diseases by IVIg treatment (14,17,19,31).

The effects of IVIg on macrophages under homeostatic conditions, or in M2-associated patho-physiological processes, had not been addressed before. Unlike M1 macrophages, treatment of M2-polarized macrophages with IVIg for 24 hours led to a significant reduction in the LPS-induced CCL2 and IL-10 release, and a concomitant enhancement of the LPS-induced production of TNFα and IL-12p40 (*Fig. 1B*). Moreover, IVIg-treated M2 macrophage supernatants, like M1 macrophage-conditioned media, inhibited the growth of BLM melanoma cells (Fig. 1C, lower panel), whose proliferation was not affected by IVIg itself. Along the same

line, IVIg provoked a dramatic transcriptomic switch in M2 macrophages, as it reduced the expression of M2-specific markers (between 5- and 100-times) and increased the expression of M1-specific markers (10-to-100-fold) (*Fig. 1D, lower panel*). Kinetic analysis revealed that the polarization switch of M2 macrophages is already evident 12 hours after IVIg addition (*Supplementary Fig. S1*). Unsupervised hierarchical clustering confirmed that the gene expression profile of IVIg-treated M2 macrophages resembles that of pro-inflammatory M1 macrophages (*Fig. 1E*). These results indicated that IVIg differentially affects the functional and transcriptomic

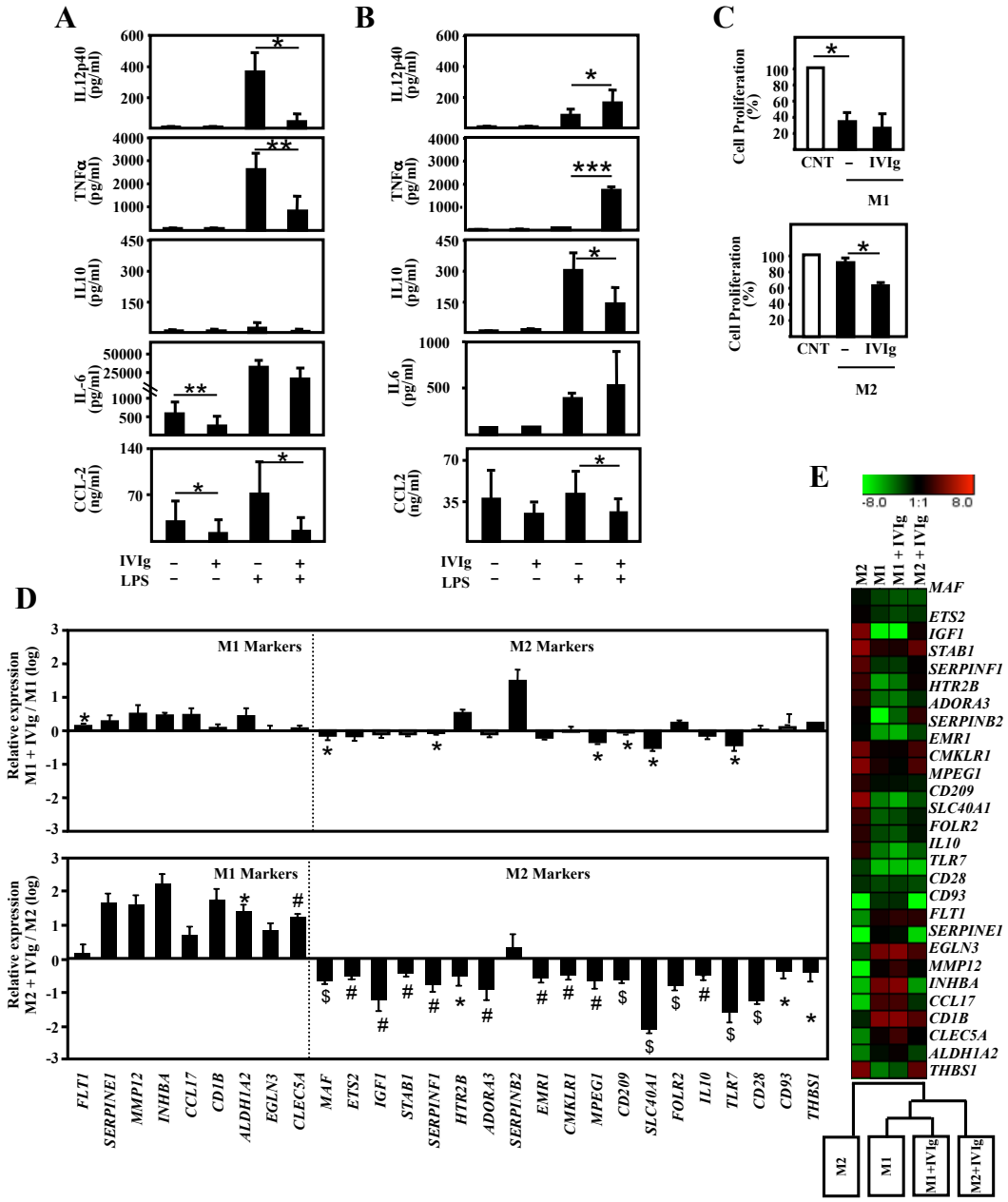

Fig. 1. Effect of IVIg on human macrophage polarization. M1 **(A)** and M2 **(B)** macrophages were cultured with (+) or without (-) IVIg (10 mg/ml, 24 hours), stimulated with LPS (10 ng/ml, 24 hours), and supernatants assayed for the indicated cytokines. Shown are the mean ± SD of four to seven independent experiments (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). **(C)** BLM melanoma cell-growth inhibitory ability of IVIg-treated M1 and M2 macrophage supernatants, relative to the proliferation of untreated BLM cells (CNT) (n=3; *, $p<0.05$). **(D)** Polarization marker expression in M1 and M2 macrophages exposed to IVIg (24 hours), as determined by qRT-PCR (n=2 for M1, n=3 for M2 macrophages). Relative Expression (log scale) indicates the expression of each marker in the presence of IVIg relative to its expression in the absence of IVIg (*, $p<0.05$; #, $p<0.01$; $, $p<0.001$). **(E)** Non-supervised hirarchical clustering (Genesis software, http://genome.tugraz.at/genesisclient/genesisclient_description.shtml) on the mean expression level of each polarization marker under the indicated culture conditions.

polarization of M1 and M2 macrophages, as it inhibits the production of pro-inflammatory cytokines by M1 macrophages while promotes the acquisition of a pro-inflammatory profile in M2 macrophages. Further supporting these results, IVIg increased the expression of most M1-specific markers, and downregulated the majority of M2-specific markers, on ex vivo isolated CD14+ human thymic macrophages (*Supplementary Fig. S2*), and reduced the constitutive and LPS-induced expression of IL-10, while potentiated that of TNFα, from CD14^{+} Tumor-Associated Macrophages (*Supplementary Fig. S2*). Altogether, these results demonstrate the ability of IVIg to promote an M2-to-M1 polarization switch in human macrophages.

CD16 and Syk phosphorylation mediates the IVIg-induced switch in macrophage polarization.- To unravel the mechanisms underlying the IVIg effect on human macrophage polarization, we initially focused on activating Fcγ receptors, and specially on CD16, whose expression is significantly higher in M2 than in

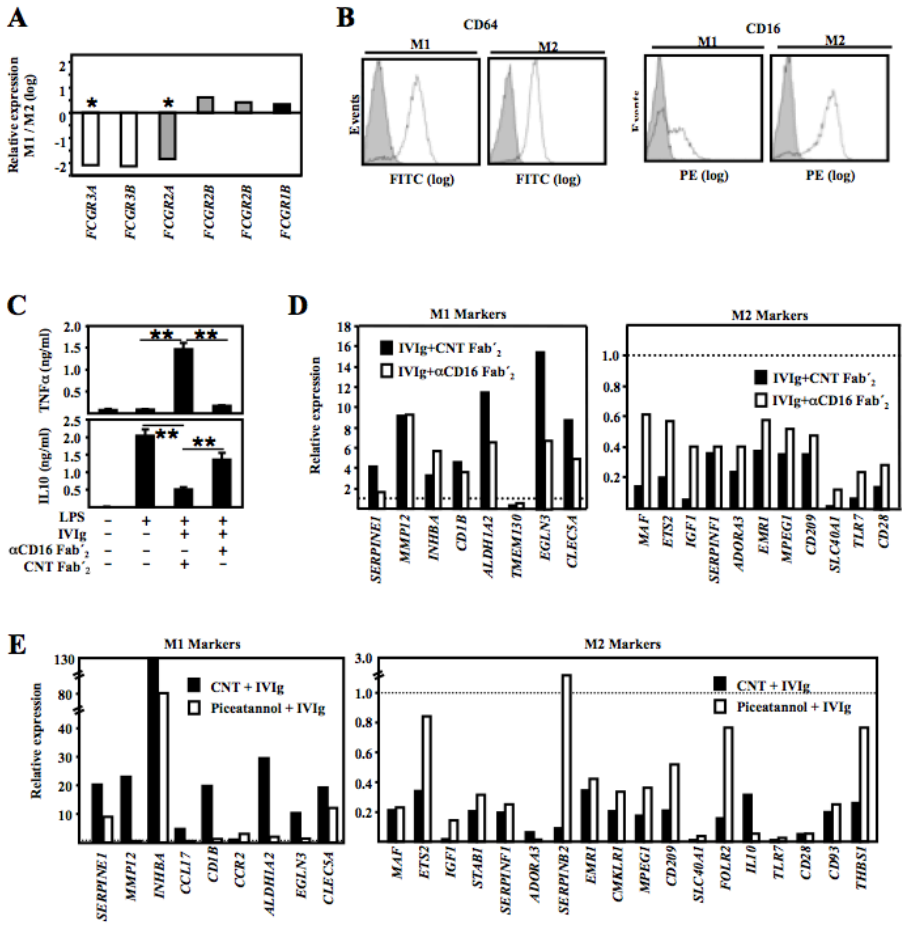

Fig. 2. CD16-Syk axis mediates the IVIg-induced M2-to-M1 human macrophage polarization switch. (A) Relative expression (log scale) of indicated FcR-encoding genes in M1 and M2 human macrophages, as determined by gene expression profiling (GSE27792, *, adjusted p<0.05). **(B)** CD16 and CD64 cell surface expression in M1 and M2 human macrophages, as determined by flow cytometry. **(C)** LPS-stimulated cytokine release from untreated or IVIg-treated M2 macrophages preincubated (30 minutes) with a blocking anti-CD16 Fab´2 antibody (αCD16 Fab´2) or an isotype-matched antibody (CNT Fab´2). Shown are the mean ± SD of three independent determinations (**, p<0.01). **(D)** M1 and M2 polarization marker expression in M2 macrophages preincubated (30 minutes) with a blocking anti-CD16 Fab´2 antibody (αCD16 Fab´2) or an isotype-matched antibody (CNT Fab´2), and exposed to IVIg (24 hours). **(E)** M1 and M2 polarization marker expression in M2 macrophages preincubated with piceatannol and exposed to IVIg (24 hours). In D and E, Relative Expression indicates the expression of each marker in the presence of IVIg and relative to its expression in the absence of IVIg (indicated by a dotted line and arbitrarily set to 1).

M1 macrophages (*Fig. 2A,B*). The contribution of the CD16-encoding FCGR3A gene to the IVIg-mediated functional polarization switch was assessed by using the anti-CD16 3G8 blocking antibody. 3G8 mAb significantly reversed the IVIg-mediated change in cytokine profile, as it abrogated the IVIg-dependent increase in TNFα production and inhibited the IVIg-mediated reduction in IL-10 release (*Fig. 2C*). Moreover, anti-CD16 impaired the IVIg-induced downregulation of M2-specific marker expressions as well as the increase of M1-specific markers (*Fig. 2D*). Taken together, these results demonstrate that CD16 is involved in the M2-to-M1 phenotypic and functional polarization switch induced by IVIg. In agreement with the known CD16-dependent Syk phosphorylation, IVIg triggered activation of Syk and its downstream targets Akt, ERK1/2, CREB and p38MAPK (*Supplementary Fig. S3*) (32,33), but had no effect on the phosphorylation state of SHIP1 (unpublished observation), a read-out for the engagement of the inhibitory CD32b Fcγ receptor (31). In fact, the IVIg-induced polarization switch was reverted by the Syk tyrosine kinase inhibitor piceatannol (*Fig. 2E and Supplementary Fig. S4*). Thus, the CD16-Syk axis mediates the M2-to-M1 macrophage polarization switch induced by IVIg in human macrophages.

IVIg modifies bone marrow-derived M2 mouse macrophage polarization via Fcer1g and Fcgr3.- To determine the extent of the relevance of these findings, we next determined whether the IVIg-mediated polarization switch was also observed in mouse bone marrow-derived macrophages (BMDM). Like in the case of human macrophages, IVIg significantly enhanced the expression of the paradigmatic M1 markers *Nos2, Tnfa* and *Cd11c*, whereas it inhibited the expression of a large number of M2 polarization markers (Fig. 3A). At the functional level, the LPS-stimulated production of TNFα by mouse M2 BMDM was significantly enhanced by IVIg, without affecting IL-10 release (Fig. 3B). The IVIg-induced polarization switch took place via Fc receptors, because ablation of the *Fcgr3* gene inhibited the increase of *Nos2* and the decrease of *Cbr2, Emr1* and *Cd206* triggered by IVIg (Fig. 3A), and because all the IVIg-triggered changes were blunted in *Fcer1g*$^{-/-}$ M2 macrophages (Fig. 3A). Regarding LPS-cytokine release, the absence of Fcεr1γ expression completely abolished the IVIg-induced increase in TNFα release from mouse M2 BMDM (Fig. 3B). Consequently, all the transcriptomic and functional changes triggered by IVIg on M2 BMDM are absent in *Fcer1g*$^{-/-}$ macrophages, whereas ablation of the *Fcgr3* gene has only a partial influence on the polarization switch triggered by IVIg. Therefore, and like in the case of human macrophages, Fcγ activating receptors mediate the pro-inflammatory polarization of mouse macrophages by IVIg.

IVIg promotes proinflammatory response in vivo.- To assess the *in vivo* relevance of the above *in vitro* data, we evaluated the influence of IVIg on three animal models of disease: a middle cerebral artery-occlusion

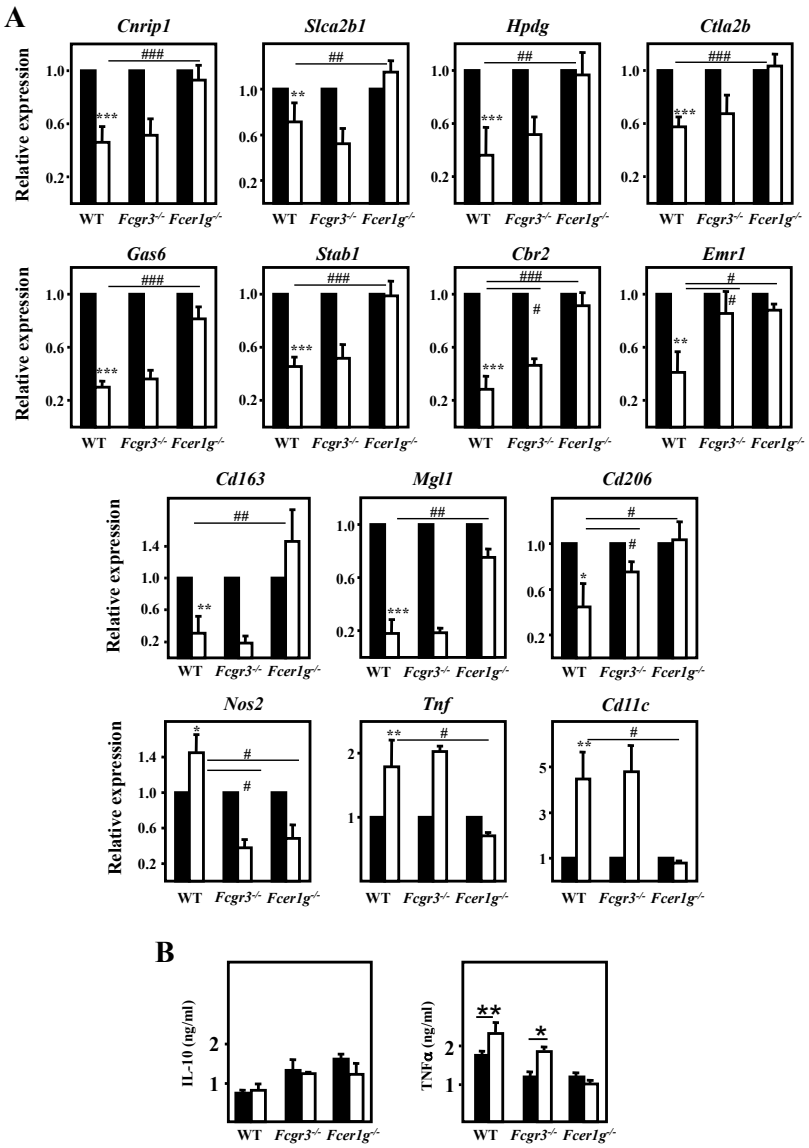

Fig. 3. Effect of IVIg on mouse macrophage polarization. (A) Polarization marker expression in untreated (CNT) or IVIg-treated (24 hours) M2 BMDM from wild type, Fcgr3-/- and Fcer1g-/- mice, as determined by qRT-PCR. Relative Expression indicates the expression of each marker in the presence of IVIg and relative to its expression in the absence of IVIg. Shown are the mean ± SD of four independent experiments (untreated vs. IVIg-treated samples: *, p<0.05; **, p<0.01; ***, p<0.001; comparison between IVIg-treated samples: #, p<0.05; ##, p<0.01; ###, p<0.001). **(B)** LPS-induced release of IL-10 and TNFα from M2 BMDM untreated or treated with IVIg (24 hours). Shown are the mean ± SD of four independent experiments (*, p<0.05; **, p<0.01).

stroke model and an LPS-induced sepsis-like mouse model, where tissue damage correlates with excessive proinflammatory responses (34), and a xenograft tumor model, where tumor and tumor-associated cells contribute to the establishment of an immunosuppressive environment (35). IVIg injection led to significant increases in the volume of the infarcted area in the stroke mouse model (*Fig 4A*), and diminished survival rates after injection of a lethal dose of LPS (*Fig. 4B*), thus implying that IVIg misbalances innate immune responses

towards a more pro-inflammatory state. Along the same line, and regarding the xenograft tumor model, IVIg significantly reduced tumor volumes after 15 days (*Fig. 4C*). Altogether, data from the three assayed animal models revealed that IVIg exerts a global proinflammatory response *in vivo.*

Inhibition of mouse tumor metastasis by IVIg is associated to changes in macrophage polarization and depends on Fcγ receptors.- Since tumor metastasis and progression are dependent on the tumor ability to alter macrophage polarization (5,36) and given the above described effects of IVIg, we hypothesized that IVIg might inhibit tumor growth and/or metastasis by skewing macrophage polarization via engagement of Fcγ receptors. In the MC38 colon cancer xenograft model, IVIg significantly reduced tumor volume in WT and *Fcgr3*$^{-/-}$ mice, but had no effect in *Fcer1g*$^{-/-}$ mice (*Fig. 5A, B*). Along the same line, IVIg significantly lowered B16F10 melanoma lung metastasis in WT mice, an inhibitory effect that was absent in *Fcgr3*$^{-/-}$ and *Fcer1g*$^{-/-}$ mice (*Fig. 5C*). Importantly, analysis of the tumor-associated CD11b+ myeloid cells from MC38 tumor xenografts revealed a significantly increased expression of the M1 polarization-associated markers *Cd11c, Ccr7* and *Nos2* in tumor-bearing WT mice but not in *Fcer1g*$^{-/-}$ mice (*Fig. 5D*). Even more, the IVIg-

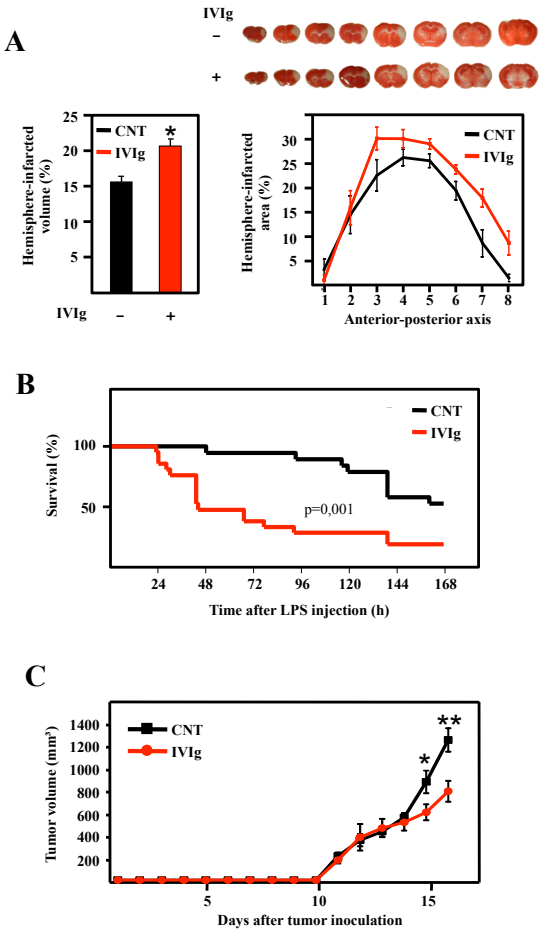

Fig. 4. Effect of IVIg on different animal models: infarct outcome after permanent middle cerebral artery occlusion, LPS-Induced Endotoxin Shock and subcutaneous tumors. (A) Infarct volume (left panel) and infarct areas (right panel) assessed by TTC staining 48 h after permanent MCAO in brain form IVIG-treated or untreated mice. The mean ± SEM is indicated. Date are pool from N=10 mice (*, p<0.05). **(B)** C57Bl/6 mice were pretreated i.p. with IVIG or PBS and one hour after injected i.p. with 350ug LPS. The survival was monitored during 7 days. Data represent mean of pool from N=20 mice**. (C)** Effect of i.v. injection of IVIg on the volume of tumors found after s.c. injection of BLM human melanoma cells in the lateral thoracic wall of BALB/c SCID mice. The mean ± SEM is indicated. Date are pool from N=8 mice (*, p<0.05).

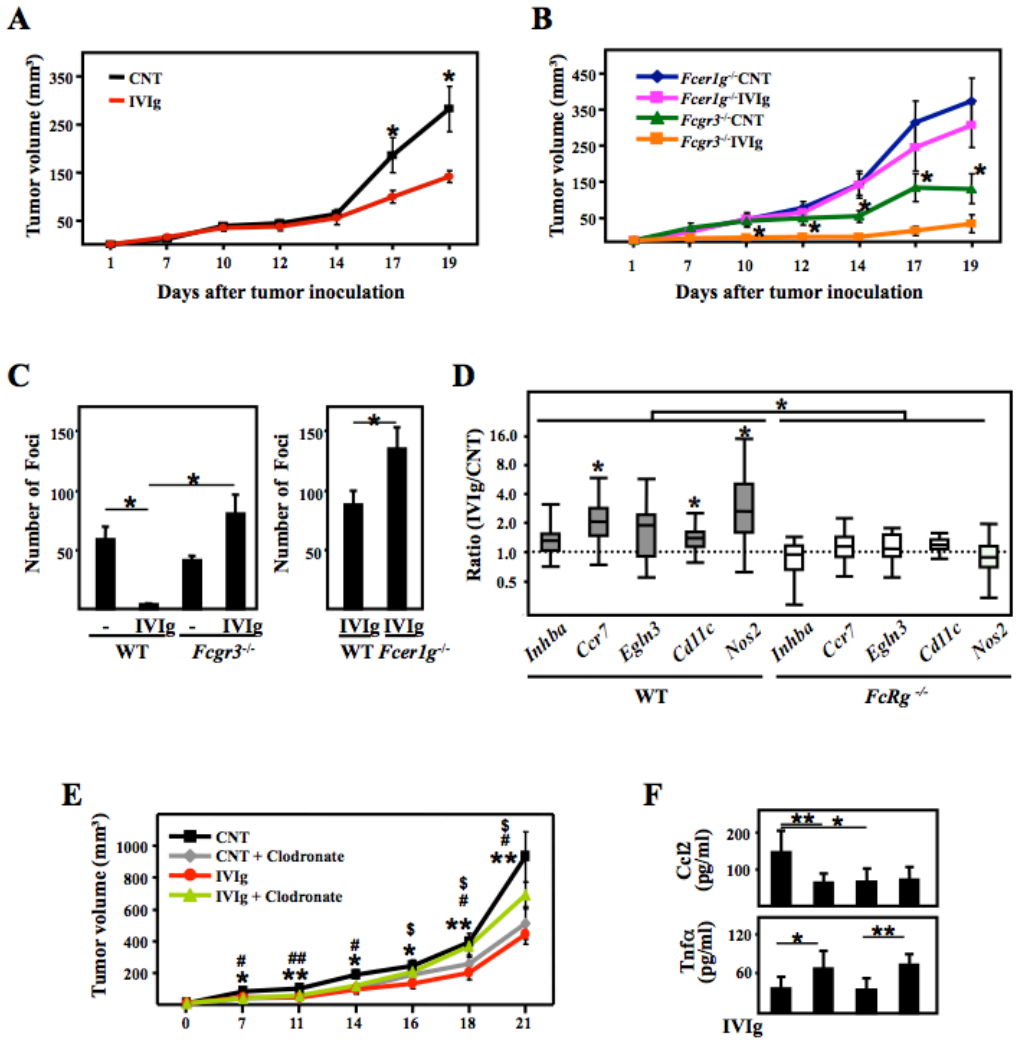

Fig. 5. IVIg inhibition of tumor progression requires macrophages and is dependent on Fcgr3 and Fcεr1γ . Tumor volume in PBS (CNT) or i.v. IVIg-treated **(A)** WT or **(B)** Fcgr3-/- and Fcer1g-/- C57BL/6 mice s.c. injected with MC38 colon cancer cells in the lateral thoracic wall. Shown are the mean ± SEM (n=7; *, p<0.05). **(C)** Number of lung metastatic foci in PBS (-) or i.v. IVIg-treated WT (n=6), Fcgr3-/- (n=6) and Fcer1g-/- (n=11) C57BL/6 mice i.v. injected with B16F10 melanoma cells. Shown are the mean ± SEM (*, p<0.05). **(D)** Polarization marker expression in CD11b+ cells isolated from tumors in PBS (CNT) or IVIg-treated (24 hours) WT and Fcer1g-/- mice s.c. injected with MC38 colon cancer cells. In all cases, the ratio of the expression level of each gene in tumor-associated CD11b+ in IVIg- vs. PBS-treated animals (IVIg/CNT) is represented (*, p<0.05). The comparison between the global expression of M1-specific markers in WT and Fcer1g -/- mice is also shown (*, p<0.05). **(E)** Tumor volume in PBS (CNT) or i.v. IVIg-treated WT C57BL/6 mice s.c. injected with MC38 colon cancer cells in the lateral thoracic wall, either with or without clodronate liposome-mediated depletion of macrophages. Shown are the mean ± SEM (n=8) (CNT vs. IVIg-treated mice: *, p<0.05; **, p<0.01; CNT vs. CNT+Clodronate: #, p<0.05; ##, p<0.01; IVIg vs. IVIg+Clodronate: $, p<0.05). (**F**) Serum cytokine levels in WT mice PBS- (-) or IVIg-treated, either with or without clodronate liposome-mediated depletion of macrophages. Shown are the mean ± SD (n=8; *, p<0.05; **, p<0.01).

mediated global upregulation of M1-specific markers group seen in WT mice was completely abolished in *Fcer1g* $^{-/-}$ mice (*Figure 5D*). Therefore, and through engagement of activating Fcγ receptors, IVIg treatment impairs tumor progression (growth and metastasis) and influences the polarization of tumor-associated myeloid cells. The causal relationship between both effects was assessed by determining the ability of IVIg to inhibit tumor growth upon macrophage depletion. As shown in *Figure 5E*, clodronate liposome-mediated depletion of macrophages prevented the IVIg-promoted tumor growth reduction in the MC38 colon cancer model. In

fact, even the decrease in serum CCL2 caused by IVIg treatment was found to be macrophage-dependent (*Fig. 5F*). Therefore, IVIg alters the polarization of macrophages, whose presence is absolutely required for IVIg to limit tumor growth.

DISCUSSION

The immunomodulatory action of IVIg has widened the range of pathologies for which IVIg therapy is either approved or has shown benefit (37,38). In line with its beneficial actions on inflammatory pathologies, we now show that IVIg impairs the effector functions of pro-inflammatory M1 macrophages. However, we also report the ability of IVIg to cause a M2-to-M1 phenotypic and functional Fc receptor-mediated polarization switch on human and murine macrophages *in vitro* and *in vivo,* thus illustrating that the IVIg immunomodulatory effects are dependent on the polarization state of the responding macrophages.

Immunocomplexes have long been known to promote tumor cell killing in an FcγR-dependent manner, and to elicit potent inflammatory responses that underlie pathologies like systemic lupus erythematosus and rheumatoid arthritis(39). From this point of view, the pro-inflammatory and anti-tumor nature of the IVIg effect that we report is not unprecedented. Paradoxically, however, high doses of IgG (IVIg) exert beneficial effects on several autoimmune disorders by virtue of their potent anti-inflammatory activity (16,40). Attempts to explain this apparent contradiction have indicated that the active components within IVIg constitute a minor fraction of the preparation (e.g., immune complexes, sialylated-Fc IgG), thus explaining the large doses requirement (14). The results that we now present shed more light on this issue, since IVIg provokes different responses, either pro- or anti-inflammatory, depending on the polarization state of the target macrophage. Whereas IVIg improves inflammatory diseases through impairment of the functional activities of M1 pro-inflammatory macrophages, the IVIg effects on M2 anti-inflammatory macrophages skews them towards the acquisition of the phenotypic and functional characteristics of M1/pro-inflammatory macrophages. These last effects are consistent with the pro-inflammatory actions exerted by immunoglobulins or immune complexes (19). Thus, considering the effects of IVIg on both types of macrophages, IVIg exhibits both pro-inflammatory and anti-inflammatory properties, and that the nature of its effects are dependent on the activation/polarization status of the target macrophages, as it enhances the pro-inflammatory activities of M2 macrophages and limits the pro-inflammatory actions of M1 macrophages. Therefore, IVIg appears as a potent and versatile immunomodulatory agent that tunes macrophage effector functions in an environment-dependent manner.

In the context of cancer, we have demonstrated that IVIg impairs tumor progression and metastasis in a Fc receptor- and macrophage-dependent manner, and that IVIg alters the expression of polarization markers in CD11b+ tumor-associated myeloid cells from WT but not *Fcer1g* $^{-/-}$ mice. The co-existence of autoimmune pathologies and cancer has previously provided evidences that IVIg therapy promotes regression of cancer in patients with chronic lymphatic leukemia, Kaposi´s sarcoma and melanoma (41). Given the importance of the polarization of tumor-associated macrophages for tumor progression and dissemination (36), our results establish a sequential link between the IVIg ability to modulate macrophage polarization and its anti-tumor effect, suggesting that the pro-inflammatory activities of IVIg might also be therapeutically useful in pathologies like cancer, where immunogenic and pro-inflammatory macrophage functions need to be promoted.

Numerous molecular and cellular mechanisms have been proposed to explain the immunomodulatory activity of IVIg (19). Our results clearly establish macrophages as an absolute requirement for the anti-tumor effect of IVIg, since macrophage depletion abrogates the inhibition of tumor growth by IVIg. Moreover, in tumor-bearing animals, IVIg inhibited the level of circulating Ccl2, whose tumor-dependent increase was significantly reduced upon clodronate-mediated macrophage depletion. This is of particular significance since CCL2 is known to promote M2 macrophage polarization (42,43) and CCL2-induced MCPIP1 inhibits proinflammatory cytokine production(44) and augments the expression of the M2-associated IL-10-driving cMaf transcription factor (26,45). The ability of IVIg to reduce CCL2 both *in vivo* (Figure 5) and *in vitro* (Figure 1A, B) might, therefore, contribute to lower the M2/anti-inflammatory environment seen in tumor-bearing animals, thus favoring the generation of anti-tumor responses. In this regard, others have also shown that IVIg decreases CCL2 levels in whole blood (46) and skin tissue (47).

Besides monocytes/macrophages, cellular targets for IVIg include NK cells, T and B lymphocytes, granulocytes and endothelial cells (48). Our data also supports the idea that cells other than macrophages contribute to the pro-inflammatory activity of IVIg because the IVIg-induced increase in circulating TNFα in tumor-bearing animals is not eliminated upon macrophage depletion (Figure 5F). Since inhibition of tumor growth by IVIg is macrophage-dependent, it seems reasonable to conclude that the IVIg-enhanced levels of TNFα do not significantly participate to the anti-tumor action of IVIg. However, and given the TNFα functional activities, it could be hypothesized that IVIg-induced TNFα might explain the mild/adverse side effects occasionally seen during IVIg therapy.

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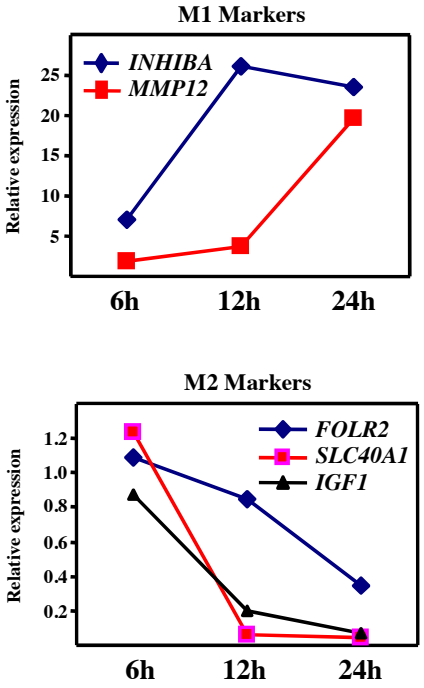
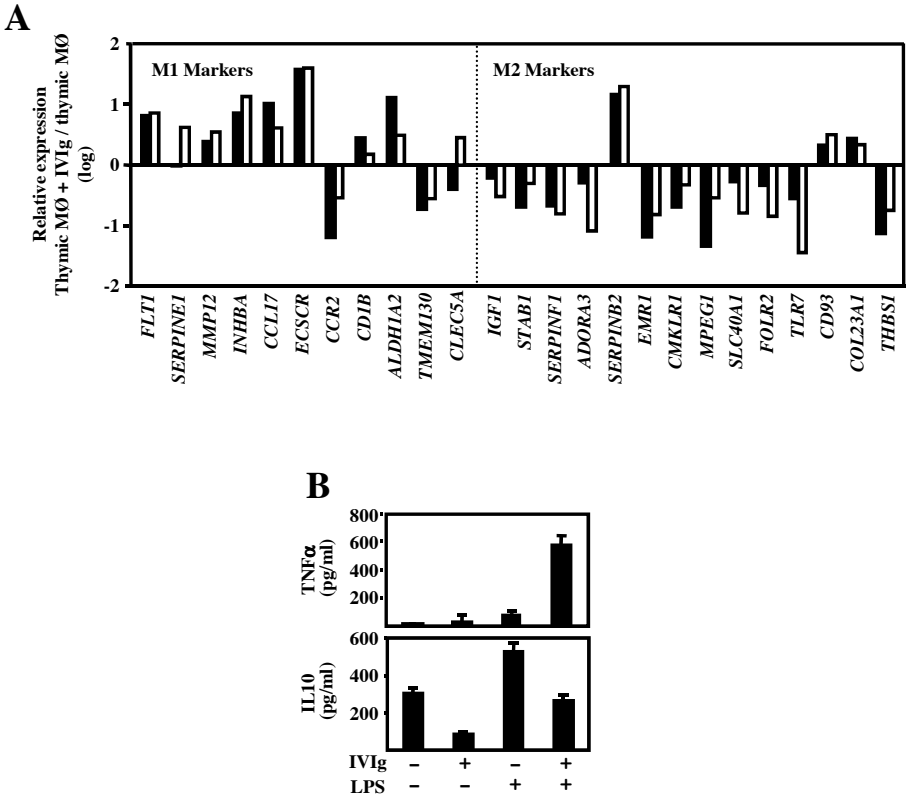
Supplementary Figure 1



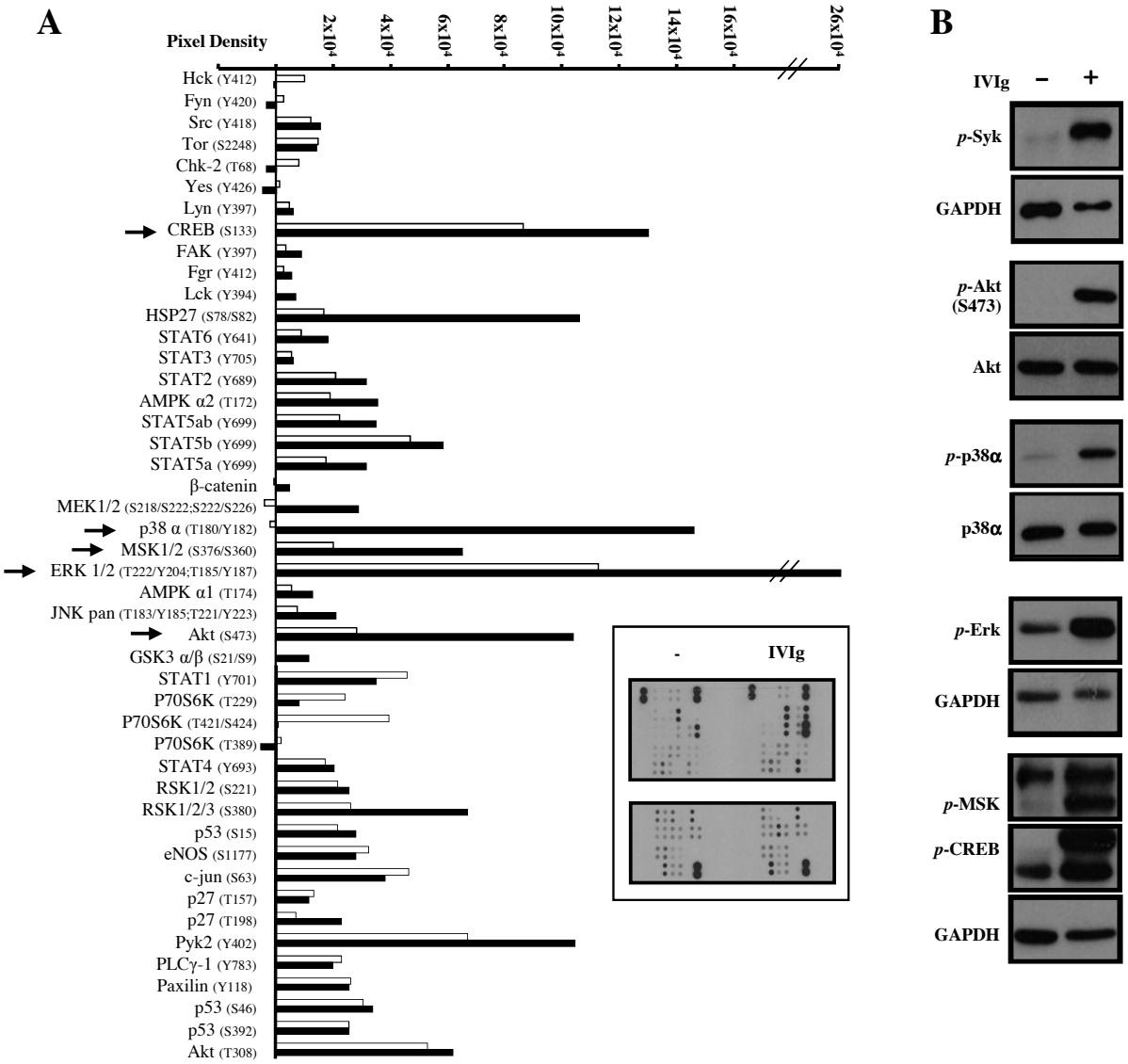
Kinetic analysis of representative M1- and M2-specific markers on IVIg-treated M2 macrophages. M2 macrophages were exposed to IVIg, and the expression level of the indicated M1 and M2 polarization markers was determined by qRT-PCR after 6, 12 or 24 hours. Results are expressed as Relative Expression, which indicates the expression of each marker in the presence of IVIg and relative to its expression in the absence of IVIg.

Supplementary Figure 2



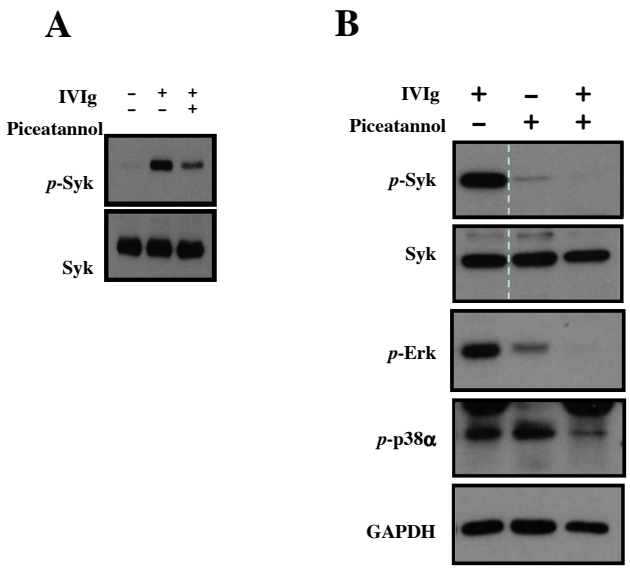
Effect of IVIg on ex vivo-isolated macrophages. (A) Thymic macrophages from two independent donors were exposed to IVIg for 24 hours, and the expression level of the indicated polarization markers was determined by qRT-PCR. Results are expressed as Relative Expression, which indicates the expression of each marker in the presence of IVIg and relative to its expression in the absence of IVIg. (B) CD14+ TAM from the pleural fluid of a metastatic breast adenocarcinoma were stimulated for 24 hours with 10 ng/ml LPS in the absence (-) or in the presence (+) of IVIg, and supernatants assayed for TNFα and IL-10. Each determination was done in triplicate and mean ± SD is indicated.

Supplementary Figure 3



IVIg-triggered intracellular signaling in human macrophages. (A) M2 macrophages were left untreated (-) or exposed to IVIg for 15 minutes, and the phosphorylation state of the indicated signaling molecules was determined using the Proteome Profiler® protein array (R&D Systems, Inc, USA), which detects the relative phosphorylation levels of 46 intracellular serine/threonine/tyrosine kinases. Results are shown as the pixel density of each spot after densitometric analysis of the blot (insert). Arrows indicate the kinases specifically mentioned in the text. (B) M2 macrophages were left untreated (-) or exposed to IVIg for 15 minutes, and the phosphorylation state of the indicated molecules was determined by Western blot using specific antibodies. Where indicated, the level of GAPDH, Akt and p38α was used as a loading control.

Supplementary Figure 4



IVIg-triggered intracellular signaling in human macrophages in the presence of Syk tyrosine kinase inhibitor piceatannol . (A) M2 macrophages were left untreated (-) or exposed to IVIg for 15 minutes either in the absence or presence of piceatannol, and the level of Syk phosphorylation and total protein state was determined by Western blot. (B) M2 macrophages were left untreated (-) or exposed to IVIg for 15 minutes either in the absence or absence of piceatannol, and the phosphorylation state of Syk, ERK1/2 and p38MAPK was determined by Western blot using phosphorylation-specific antibodies. Where indicated, the level of Syk and GAPDH was used as a loading control.

DISCUSSION

Serotonin and M2 polarization

Macrophage polarization is critically determined by the cytokine environment and dictates the range of effector functions of macrophages within a tissue (220). M1 and M2 macrophages exhibit different functions and, display opposite effects in some scenarios (220). Thus, the identification of specific markers that properly distinguish M1 and M2 macrophages should provide potentially useful therapeutic targets for pathologies where altered or deregulated macrophage polarization takes place. In the present work we demonstrate that 5-HTR_{2B} and 5-HTR_{7} serotonin receptors are preferentially expressed by *in vitro* generated M2(M-CSF) macrophages, and that two well-known populations of *ex-vivo* M2 macrophages, (Kupffer cells and Tumor Associated Macrophages, TAM) express 5-HTR_{2B} at the mRNA and protein levels. Indeed, our results reveal that 5-HT modulates macrophage functions and phenotype by acting through both receptors. 5-HT skews macrophages towards an M2 phenotype by inducing a set of M2-marker genes and repressing the expression of several M1 markers. Even more, blockade of these two 5-HT receptors during the M-CSF-derived macrophage polarization *in vitro* partially impairs this process, further confirming that 5-HT contributes to the M-CSF-mediated macrophage polarization.

Detailed analysis of the M1 and M2 macrophage gene expression profiles revealed that 6 hour-treatment with 5-HT, or with the 5-HTR_{2B} agonist BW723C86, induces significant changes in the expression of a number of M1- and M2-specific genes, including *MS4A7, TTYH2, ISG20, RGS2, OLFML2B, HMOX1, S1PR1* and *NAMPT,* thus confirming the involvement of 5-HTR_{2B} in polarization towards an M2-like state. However, those experiments also revealed that 5-HT-mediated gene expression changes are mostly dependent on 5-HTR_{7}. More specifically, our data showed that most of the transcriptional effects induced by 5-HT on macrophages are mediated by the 5-HTR_{7}/PKA axis, since 5-HTR_{7} agonists or antagonists either reproduce or neutralize the majority of 5-HT-mediated changes. In this regard, it has been previously described that cAMP leads to an M2 polarization (25). Thus, GM-CSF-derived macrophages exposed to PDE inhibitors or cAMP inducers enhanced the expression of *CD163, IL-10* and a number of wound healing-related markers (a function typically associated to M2 polarization) (221). Moreover, IL-10 expression is mainly controlled by CREB, whose activity is directly controlled by cAMP (2). Altogether, these observations confirm that 5-HT, via activation of the 5-HTR_{7}/cAMP/PKA/CREB axis, and in combination with 5-HTR_{2B}-mediated pathways, participates in shaping an M2-like polarization in macrophages.

Of interest, it has been described that platelet-derived serotonin delays virus clearance in a model of hepatitis C viral infection (222). These observations might be partially in agreement with ours, since M1 (IFNγ-activated) macrophages could participate in virus clearance, and we have shown that serotonin skews macrophages polarization towards an M2-like state. However, we have also illustrated the existence of a link between 5-HTR_{2B} -initiated signals and the type I IFN signalling pathway. The observation of increased *IFNB* mRNA levels just two hours after BW723C86 treatment, and the subsequent induction of IFN-dependent genes (*CXCL10, ETV7, IFIT1, IFIT2, RSAD*), leads us to propose a role for 5-HTR_{2B} in infectious pathologies and, therefore, its potential use as a therapeutic target. However, at present, it remains to be determined whether 5-HT modifies the expression of IFN-target genes, or can directly promote IFN synthesis and release. Although preliminary ELISA experiments indicate that 5-HT is not able of triggering IFNβ released by it self, more extensive analysis are required to completely rule out this possibility.

5-HT AND INFLAMMATION

It has been reported that 5-HT modulates the cytokine profile in mononuclear cells, monocytes, monocyte-derived dendritic cells and *ex vivo* isolated macrophages (Table 2). Of interest, many of those previous observations were done in serum-containing media, where 5-HT concentration is as high as 1-2 μM (223). In the majority of those cases, the levels of LPS-induced TNF-α were reduced upon stimulation with 5-HT. Nevertheless, the correlation between 5-HT stimulation and the levels of pro-inflammatory (IL-6 or IL-12) or anti-inflammatory cytokines (IL-10) remained unclear in those studies, and the variability in the reported data has been attributed to differences in cell type or signalling through distinct 5-HT receptors. We believe that the high level of 5-HT in serum, as well as the presence of serum factors that could modulate or synergize with 5-HT, might have precluded an accurate determination of the influence of 5-HT. Therefore, in an attempt to avoid these potential artefacts, we decided to maintain macrophages in serum-free media for 48 hours before assaying the functional or transcriptional effects of 5-HT.

In these conditions, we observed that a co-stimulation with 5-HT/LPS inhibits the LPS-stimulated TNFα and IL-12p40 production, but has no effect on IL-10, thus shifting the balance towards a less pro-inflammatory response. We also found that a 5-HT pre-treatment altered the LPS-induced cytokine response towards a diminished production of pro-inflammatory cytokines. In all cases, we have determined that the 5-HT-mediated

References	Cell type	Stimuli	LPS	Cytokine effects	Medium
Life Sci. 48:2557, 1991	**Human monocytes**	Serotonin	Yes	Decreased TNF-α	
Int. Immunol. 15: 233, 2003	**PBMCs** (whole blood)	Serotonin DOI ($HTR2_{A/C}$ agonist)	Yes	Decreased TNF-α No effect on IL-10	RPMI+5% human serum
J. Immunol. 172: 6011, 2004	**Dendritic cell** (GM-CSF + IL-4)	Serotonin DOI ($HTR2_{A/C}$ agonist) Agonists for HTR4 and HTR7	Yes	Decreased TNF-α and IL-12p70 Enhaced IL-1 and IL-8	RPMI + 10% FBS
Psychiatry Res. 134:251, 2005.	**Whole blood**	Serotonin mCPP ($HTR2_{C/B}$ agonist, partial for $HTR2_A$)	Yes	Decreased TNF-α and IL-6 with serotonin. Increased with mCPP	Not specified
Int. Immunol. 17: 599, 2005	**Human monocytes** ($CD14^+$)	Serotonin DOI ($HTR2_{A/C}$ agonist) HTR4 and HTR7 agonists	Yes	Decreased TNF-α (HTR4 and HTR7), Increased IL12p40 (HTR4 and HTR7) and IL-1β, IL-6, IL-8 by serotonin. HTR2 agonists did not modulate LPS-induced cytokine production	RPMI + 10% FBS
Clin. Exp. Immunol. 146: 354, 2006	**Dendritic cell** (GM-CSF + IL-4 ± Serotonin)	Serotonin Inespecific agonist/antagonist	Yes	Increased TNF-α, IL-10, IL-6, IL-8	RPMI +10% FBS
J.Invest. Dermatol. 2007 Aug; 127(8):1947-55	**Human monocytes**	Serotonin	Yes	Increased TNF-α and IL-6 No effect on IL-10	No FBS for 1h during 5HT stimulation and then RPMI 10% FBS
Clin. Exp.Immunol. 150: 340, 2007	**NR8383** (rat alveolar cell line) **Human alveolar macrophages** (purified by a 2h adherence step)	Serotonin	Yes	(weak changes) Decreased TNF-α Increased IL-10	RPMI + 5% FBS
Rheumatol. Int. 28:1017, 2008.	**Human Synovial macrophages**	Serotonin	No	Increased PGE2	IMDM + insulin
Int J Neuropsychopharmacol. 12:525, 2009	**Peritoneal macrophages** (thioglycollate)	Venlafaxine (inhibitor of 5-HT re-uptake)	Yes	Decreased TNF-α and IL-6	RPMI + 10% FBS
Arthritis Rheum 62:683, 2010	**Human Synovial membrane**	Serotonin	No	No efects on IL-6 or TNF-α producción	RPMI + 10% FBS
Am J Physiol Lung Cell Mol Physiol 299:L272, 2010.	**Murine Alveolar macrophages**	Serotonin	No	Increased CCL2	RPMI + 0.1% FBS
Am. J. Pathol. 178:662, 2011	**Murine Gut Dendritic cell**	Serotonin	Yes	Increased IL-12	

Table 2. serotonin modulates cytokine profile in several mononuclear cells.

anti-inflammatory action is 5-HTR_7-dependent, as pre-treatment with a 5-HTR_7-specific antagonist reverted the 5-HT-dependent inhibition, whereas 5-HTR_{2B} modifiers had no effect on cytokine production. Therefore, the anti-inflammatory action of 5-HT in terms of cytokines production seems to be exclusively 5-HTR_7-dependent.

In line with our results on *in vitro* generated macrophages, it has been reported that 5-HT, via 5-HTR_7, negatively regulates TNF-α secretion from mature MDDC (186) and LPS-activated monocytes (185). Here we extend those observations to M2(M-CSF) macrophages. In the case of resolution-phase macrophages, a unique macrophage population is capable of secreting pro-inflammatory cytokines at early stages, and anti-inflammatory cytokines later in the process (25). This particular switch is mediated by cAMP, which inhibits TNFα production without affecting IL-10 release (25). Of importance, as mentioned above, we have found a similar effect on the cytokine profile through activation of the 5-HT_7-cAMP-PKA axis. Therefore, our results suggest that 5-HT might contribute to inflammation resolution by binding to 5-HT_7, and that cAMP may be the key intracellular molecule that mediates the 5-HT-promoted inhibition of TNFα release.

Regarding 5-HTR$_7$, it has been reported that specific agonists of this receptor favour the release of IL-1β and IL-8 on both MDDC (186) and monocytes (185). Along this line, we have observed that macrophages pre-treated with either 5-HT or BW723C86 up-regulate IL-1β mRNA, and that higher levels of LPS-induced IL-1β are released from 5-HT-treated macrophages. We postulate that, although IL-1β acts as a pro-inflammatory cytokine, its enhanced expression after 5-HT treatment might also exert an inhibitory feed-back activity, as it might increase the expression and activity of the 5-HT transport (SERT) (180) on platelets (224), thus facilitating 5-HT removal.

Given the net anti-inflammatory action that 5-HT displays, and in line with the increase of *IL1B* mRNA triggered by 5-HT, the induction of *TREM-1* mRNA levels by 5-HT was also unexpected, because *TREM-1* potentiates macrophage activation (225) and favours the production of TNFα, IL-6 and IL-8 by colon macrophages (226). However, soluble forms of TREM family members (sTREM) have been described (225), and whose generation has been explained by either alternative splicing (227) or metalloproteinase-processing of TREM extracellular domains (228). Little is known about sTREM-1 functions, but it is thought to negatively regulate *TREM-1* receptor signalling by neutralizing its ligands (225). In fact, it has been proposed that sTREM-1 levels correlate positively with IL-10 and negatively with IL-8 in chronic colitis patients (229). Therefore, we hypothesize that the enhanced levels of *TREM1* mRNA observed after 5-HT treatment might give rise to sTREM-1, an hypothesis that we are currently testing. If so, the modulation of TNFα secretion by 5-HT could be partly mediated via sTREM-1.

Altogether, our results extend previous findings on the anti-inflammatory capacity of 5-HT, and provide evidences that 5-HTR$_7$ is responsible for the change in cytokine profile provoked by 5-HT on human macrophages.

5-HT-INDUCED CELL PROLIFERATION

5-HT regulates cell proliferation in various cell types, including hepatocytes (230) and tumor cells (231). On the other hand, macrophages are able to secrete several proliferative factors such as EGF (232), vascular endothelial growth factor (VEGF) (2, 233), platelet derived growth factor (PDGF), hepatocyte growth factor (HGF) (234) or TGF (2). Our results on the 5-HT-controlled gene profile in macrophages have identified

epiregulin (*EREG*), a member of the EGF family of growth factors (235), as a 5-HT-inducible factor, and suggest that it might contribute to the control of cell proliferation by 5-HT as we will discuss next.

The link between 5-HT and cell proliferation has been known for a long time, and is more evident in organs like heart and liver, where 5-HT levels have a clear physiopathological influence. The adult mammalian liver is a highly regenerative organ (230). It is capable of rapidly and effectively restoring loss of mass and rebuilding complex tissue structures, such as hepatic sinusoids and bile conducts, that are vital for normal liver function (236). Both liver regeneration and hepatocyte proliferation are regulated by 5-HT. $TPH1^{-/-}$ hepatectomized mice (where lack of TPH1 impairs the 5-HT synthesis in peripheral tissues) display a robust reduction of several markers associated to hepatocyte proliferation (237), suggesting that 5-HT is involved in the induction of liver regeneration. Of interest, the knock out of SERT in rats, where the 5-HT concentration in platelets is very low, showed that 5-HT derived from platelets might not be involved in the proliferative process of the liver regeneration (238), that might be exclusively mediated by enterochromaffin cell-derived 5-HT (238) or 5-HT from epithelial cells in the bile conduct (Cholangiocytes) (239). Interestingly, activation of 5-HT_{2B} improves animal survival in small liver grafts transplantations (240) and age-associated impairments in regenerative capacity (241), further illustrating the involvement of this receptor on 5-HT-promoted cell proliferation.

We have found that 5-HT_{2B} receptor is expressed on Kupffer cells, localized in liver sinusoids and continuously exposed to circulating serotonin in peripheral blood. Kupffer cells play a key role in liver regeneration and repair through the secretion of numerous cytokines that participate in both processes (242). Therefore, it is temping to speculate that the presence of 5-HT_{2B} on Kupffer cells might have an impact on liver regeneration. Such hypothesis would be supported by the fact that 5-HT and BW723C86 favour the in vitro maintenance of the M2 macrophage polarization state (which is characterized by its tissue-repair and cell growth- promoting properties), as well as by the 5-HT-induced production of the growth-promoting factor epiregulin. Thus, the role that both Kupffer cells and 5-HT play on liver regeneration (and therefore on hepatocyte proliferation) might be in part mediated by the 5-HT-inducible growth factor *EREG*.

Also related to the link between serotonin and cell growth, the detection of 5-HT_{2B} in immunosuppressive tumor-associated macrophages (TAMs) (2) suggests the contribution of serotonin to tumor development (fig. 12). Besides the fact that serotonin may directly foster tumor cell proliferation in some cases (243-245), the

growth of murine colon cancer allografts is dependent on the 5-HT-mediated reduction in MMP12 levels by TAMs, that leads to enhanced angiogenesis (179). Our results are in concordance with these observations, since we have demonstrated that 5-HT, or BW723C86, promotes a down-regulation of *MMP12* mRNA levels. Moreover, *EREG* is up-regulated upon 5-HT, BW723C86 or LP-12 (a specific 5-HT_7 agonist) treatments in just two hours. EREG promotes tumor cell proliferation in several cancer models (235, 246), and EREG levels are induced in human patients with colitis-associated cancer (246). It has also been shown that monocyte-derived EREG has pro-tumoral effects (235). Therefore, it can be speculated that 5HT-induced EREG up-regulation can facilitate tumor cell proliferation and metastasis. Along the same line, the presence of sTREM-1, potentially induced by 5-HT in macrophages, correlates with a poor prognosis in patients with non-small cell lung cancer (247). Altogether, these observations might contribute to better define the involvement of M2 macrophages and serotonin in tumor progression, suggesting that the pro-tumoral actions of M2 macrophages might be explained, at least in part, by the increased expression of 5HT-induced factors such as EREG and TREM-1 (fig. 12).

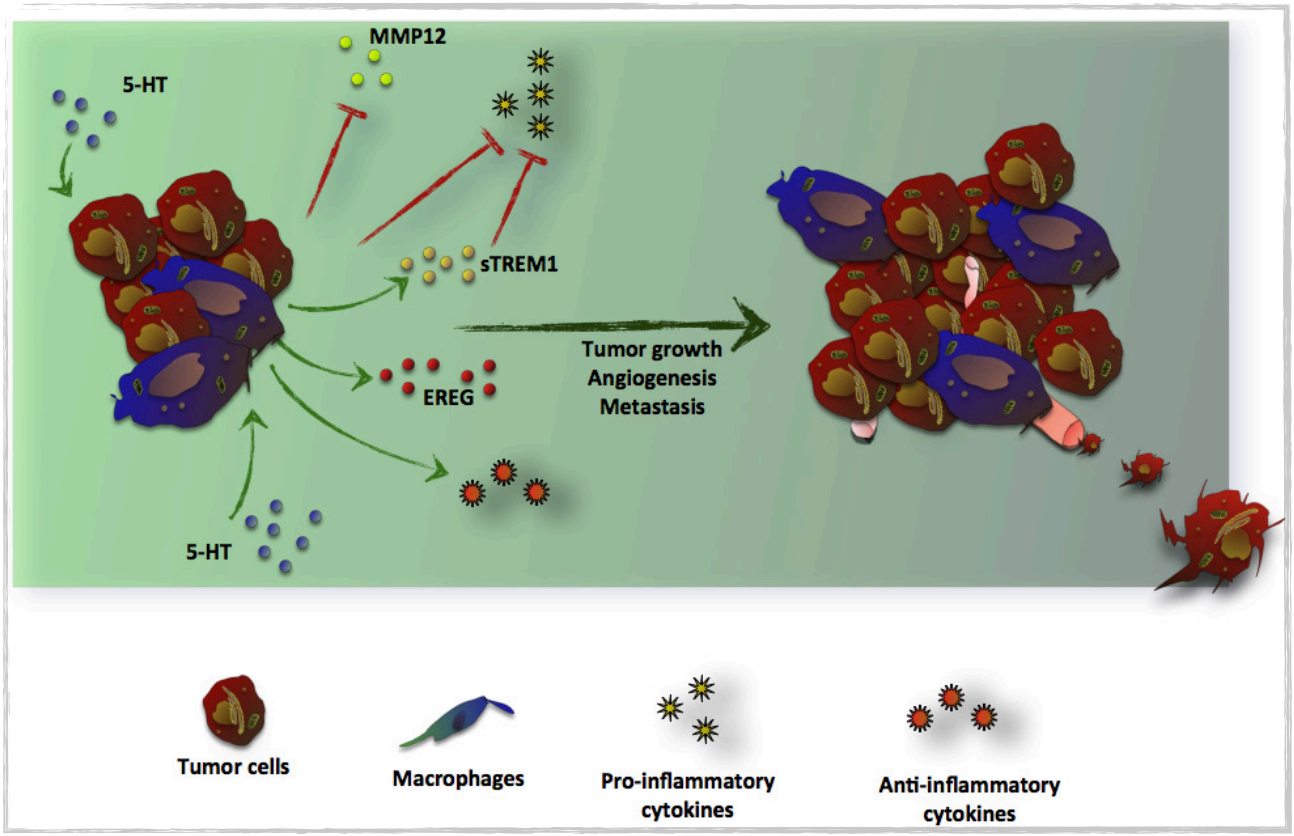


Figure 12. Possible role of serotonin on macrophage-dependent tumor promotion.

Pathologies dependent on 5-HT_{2B}, such as pulmonary arterial hypertension (PAH) or valvular heart disease (VHD), are associated with abnormal proliferation of vascular smooth muscle cells (135) or valvular fibroblasts

(131), respectively. In line with the potential effect of 5-HT-induced epiregulin in liver regeneration and tumor promotion, epiregulin might be also involved in the proliferation of smooth muscle cells and fibroblasts that underlie both pathologies. Moreover, in the specific case of PAH, it has been described that 5-HT contributes to the pathogenesis through 5-HT_{2B} in bone marrow-derived cells (140) (a group of cells that includes macrophages). Besides, several observations point to the possible link between PAH and IFN (248), as type II IFN-γ, type I IFN-α and IFN-β activate pulmonary vascular cells to release the vasoconstrictor and pro-mitogen hormone endothelin-1 (ET-1), that enhances PAH (248). In addition, a positive correlation has been established between raised levels of CXCL10 (IP-10) and PAH in patients with systemic sclerosis (249) and, in fact, IFN-β1–treated multiple sclerosis patients develop PAH (250, 251). Our gene expression results have revealed that stimulation of 5-HT_{2B} with the specific agonist BW723C86 enhanced the mRNA levels of *IFNB* and, moreover, the levels of several IFN-dependent genes, including *CXCL10*. Therefore, we can speculate that macrophages might play an important role in the development of PAH, as stimulation of 5-HT_{2B} might lead to enhanced levels of both *EREG* (that could promote vascular proliferation) and *IFNβ/CXCL10* (that might exacerbate PAH development). Consequently, our gene expression data have allowed the definition of a link between 5-HT and IFN that might be of pathological significance in the case of PAH (fig.13).

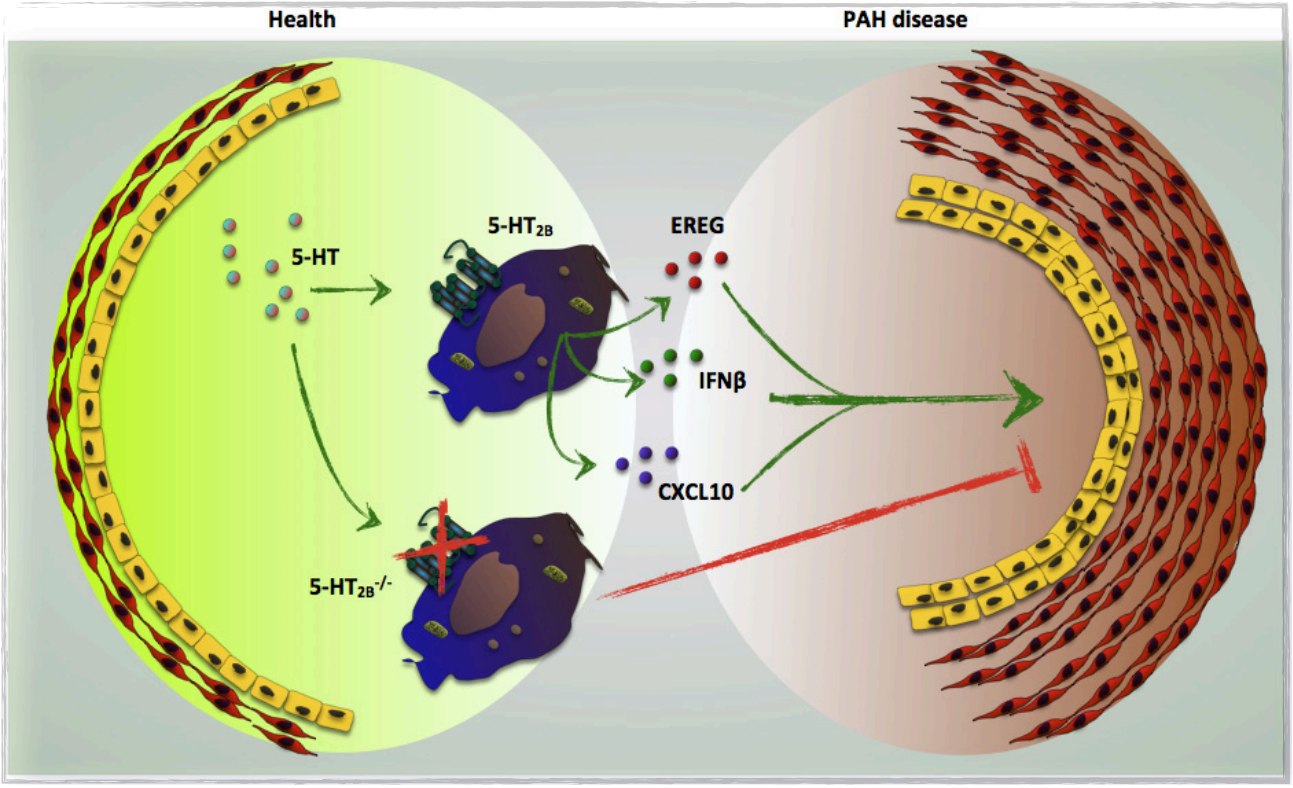


Figure 13. Possible role of serotonin on macrophage-dependent PAH.

In summary, given the implication of 5-HT in the abnormal cell proliferation that takes place in the aforementioned pathologies, we can hypothesize that the 5-HT-inducible growth factor epiregulin might lie at the basis of the deleterious cellular proliferation seen in PAH and other 5-HT-associated pathologies.

Serotonin and Wound Healing

M2 macrophages play an important role in wound healing. In this process, platelets and serotonin are well-defined players (236), so it is tempting to speculate that serotonin released from platelets might directly modulate macrophage functions during wound healing. Wound healing is a highly complex and coordinated process that can be separated in four distinct phases after tissue injury:

1. Coagulation and homeostasis.
2. Inflammation.
3. Fibro-proliferative process (can eventually be resolved over several weeks or months)
4. Full restorative regeneration of functional tissue.

At initial stages, platelet aggregation at the damaged tissue leads to the release of 5-HT, which then contributes to homeostasis due to its vasoactive properties (236). Rapidly, in the following steps, macrophages orchestrate the inflammation process by producing high doses of pro-inflammatory cytokines in response to potentially damaging exogenous or altered endogenous products (236). Subsequently, and taking our results into account, platelet-derived serotonin might act on macrophages to switch their cytokine profile, thus reducing the production of pro-inflammatory cytokines (such as TNFα and IL12p40). If so, this event could break the balance towards an anti-inflammatory condition, and would lead to a serotonin-mediated inflammation resolution.

In parallel, 5-HT would also stimulate *EREG* secretion, thus favouring fibroblast and endothelial cell proliferation for restoring tissue integrity and functionality. At a later step, the activation of the serotonin transport (SERT) by IL-1β, itself an 5HT-inducible cytokine, could represent a mechanism to remove serotonin from the serum and to help recovering tissue homeostasis (fig14).

Altogether, our data demonstrate that 5-HT_{2B} and 5-HT_{7} are crucial receptors that mediate the skewing effect of serotonin on human macrophage polarization, and might also regulate the serotonin-dependent pro-proliferative and tissue-repair activity of macrophages during physiological and pathological processes.

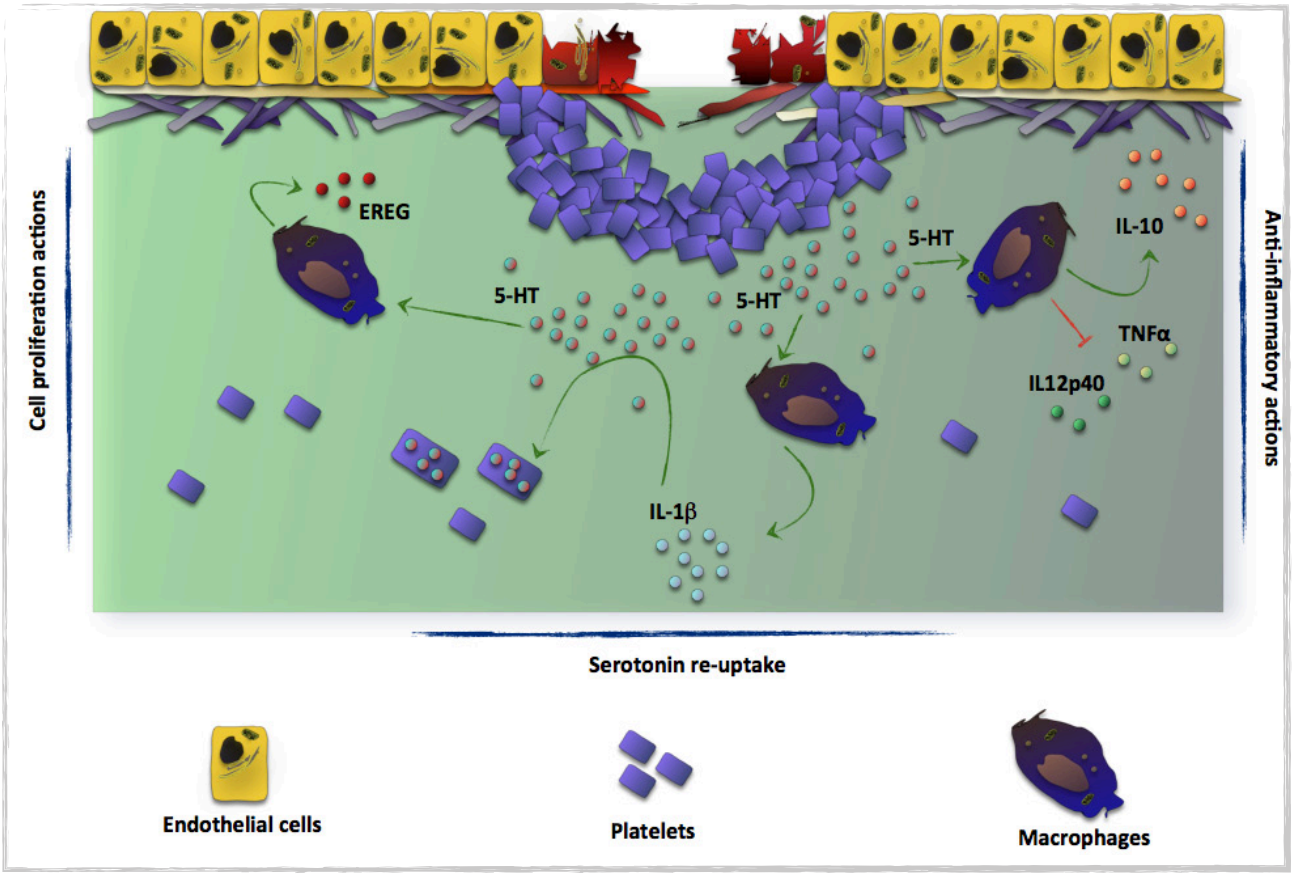


Figure 14. Possible role of serotonin and macrophages in wound healing.

IVIg and Macrophages Polarization

The immunomodulatory action of IVIg has widened the range of pathologies for which IVIg therapy is either approved or has shown benefit (194, 195). Due to its beneficial actions on inflammatory pathologies and its ability to limit tumor metastasis (202, 203), we hypothesized that IVIg might also exert its therapeutic action through modulation of the macrophage polarization state, whose M2-skewing contributes to physiological (inflammation resolution) and pathological (tumor progression and metastasis) processes. We have demonstrated that IVIg, via activating Fc receptors, causes an overt M2-to-M1 phenotypic and functional polarization switch in human and murine macrophage both *in vitro* and *in vivo*. Besides, in murine tumor models, we showed that IVIg treatment reduces tumor progression and metastasis, and alters the expression of polarization markers in $CD11b^{+}$ tumor-associated myeloid cells. Given the importance of tumor-associated macrophages for tumor progression and dissemination (252), our results establish a sequential link between the IVIg ability to modulate macrophage polarization and its anti-tumor effect, suggesting that the pro-inflammatory activities of IVIg might have therapeutic relevance.

The beneficial effects that, by virtue of their anti-inflammatory activity, high doses of IVIg exert on several autoimmune disorders (253, 254), are in concordance with our finding that IVIg-treated M1-macrophages exhibit lower LPS-stimulated production of pro-inflammatory cytokines. This indicates that IVIg exert anti-inflammatory actions within a pro-inflammatory context. However, since IVIg was not able to overtly alter the expression of polarization-associated genes, the effect of IVIg on M1 macrophages cannot be really defined as a switch in the macrophage polarization state.

A different picture emerged when analysing the influence of IVIg on M2 macrophages. IVIg administration to M2-macrophages led, in fact, to opposite results. In this anti-inflammatory (and hence pro-tumoral) context, IVIg exhibited pro-inflammatory and anti-tumoral actions, as it modulated not only the cytokine profile (enhancing pro-inflammatory and reducing anti-inflammatory cytokine release), but also the balance of M1 and M2 polarization markers: whereas IVIg turns off M1 macrophage functional activities, it promotes an M2-to-M1 polarization switch at the transcriptomic and functional level. In essence, and considering the somewhat opposite effects of IVIg on both types of macrophages, it appears that IVIg is capable of exerting both pro-inflammatory and anti-inflammatory properties, and that the nature of its effects is critically dependent on the activation/polarization status of the target macrophages (enhancing pro-inflammatory activities of M2 macrophages and limiting the pro-inflammatory activity of M1 macrophages).

IVIg in tumor promotion and metastasis

Tumors are not just a mass of genetically abnormal proliferating cells, but are now considered as a heterogeneous and structurally complex tissue (255). Malignant tumor cells recruit a variety of cell types, including fibroblasts, immune inflammatory cells, and endothelial cells, through the production and secretion of stimulatory growth factors, chemokines and cytokines (256). This assortment of cells and molecules comprises the tumor microenvironment. At later states of the tumor development, anti-tumor immunity within the tumor microenvironment can be suppressed by a variety of tumor infiltrating leukocytes, including regulatory T cells (Treg) (257), myeloid-derived suppressor cells (MDSC) and M2-macrophages (252, 258). The mechanisms employed by both malignant cancer cells and the immune cell types to suppress effective immunity include secretion of cytokines such as TGFβ and IL-10 (252), that promote a Th2 immune response, less effective against tumor cells (259).

Macrophage plasticity opens the possibility of manipulating tumor-associated immunosuppressive M2 macrophages to become immuno-stimulatory M1-like macrophages (2). Several strategies that combine chemotherapy with specific antibodies (e.g. anti-IL-10 (103), anti-CD40 (260)) synergize in promoting anti-tumoral effects and are associated with re-polarization of the infiltrating tumor-associated macrophages. These macrophage re-polarization would be able to, at least partially, change the tumor microenvironment by secreting pro-inflammatory cytokines and chemokines and promote the triggering of a Th1, and therefore anti-tumoral, type of immune response. Thus, re-programming immunosuppressive myeloid cells (in combination with other therapies) constitutes a good strategy against tumor development, and IVIg represents an attractive tool for such a purpose.

The co-existence of autoimmune pathologies and cancer has provided evidences that IVIg therapy promotes regression of cancer in patients with chronic lymphatic leukemia, Kaposi´s sarcoma and melanoma (203). These findings, together with the effect of IVIg on tumor metastasis in murine cancer models, have led to the proposal of IVIg as a potential anti-metastatic agent (203). This anti-metastatic effect has been explained by others by either direct effects on endothelial cells (inhibition of NFκB activation) (261), actions on innate immune cells like cell cycle arrest (262), induction of IL-12 secretion (263) or inhibition of MMP9 mRNA expression (264). Our results point to "modulation of macrophage polarization" as an additional mechanism to explain the IVIg´s ability to limit tumor growth and metastasis.

Macrophages are essential cells for tumor progression as they secrete pro-metastatic and pro-angiogenic cytokines, chemokines and growth factors into the microenvironment (fig.15). In the present work we have demonstrated that IVIg is capable of switching M2 pro-tumoral macrophages into M1 anti-tumoral macrophages, thus favouring the pro-inflammatory actions necessary to confront tumor growing and metastasis (fig.15). We have also demonstrated that the anti-tumoral effect of IVIg is exclusively mediated by macrophages.

Moreover, the detailed analysis of the genetic profile that governs the IVIg-evoked M2-to-M1 polarization process revealed that IVIg induces a remarkable decrease in several M2-polarization markers that are expressed by TAMs, such as *CD209* (67), *FOLR2* (65) and *HTR2B*. Our group has previously reported that DC-SIGN triggers macrophage IL-10 production upon interaction with DC-SIGN ligands (e.g., CD15) on tumor cells (67). Therefore, we can hypothesize that the IVIg-evoked decrease of DC-SIGN could reduce

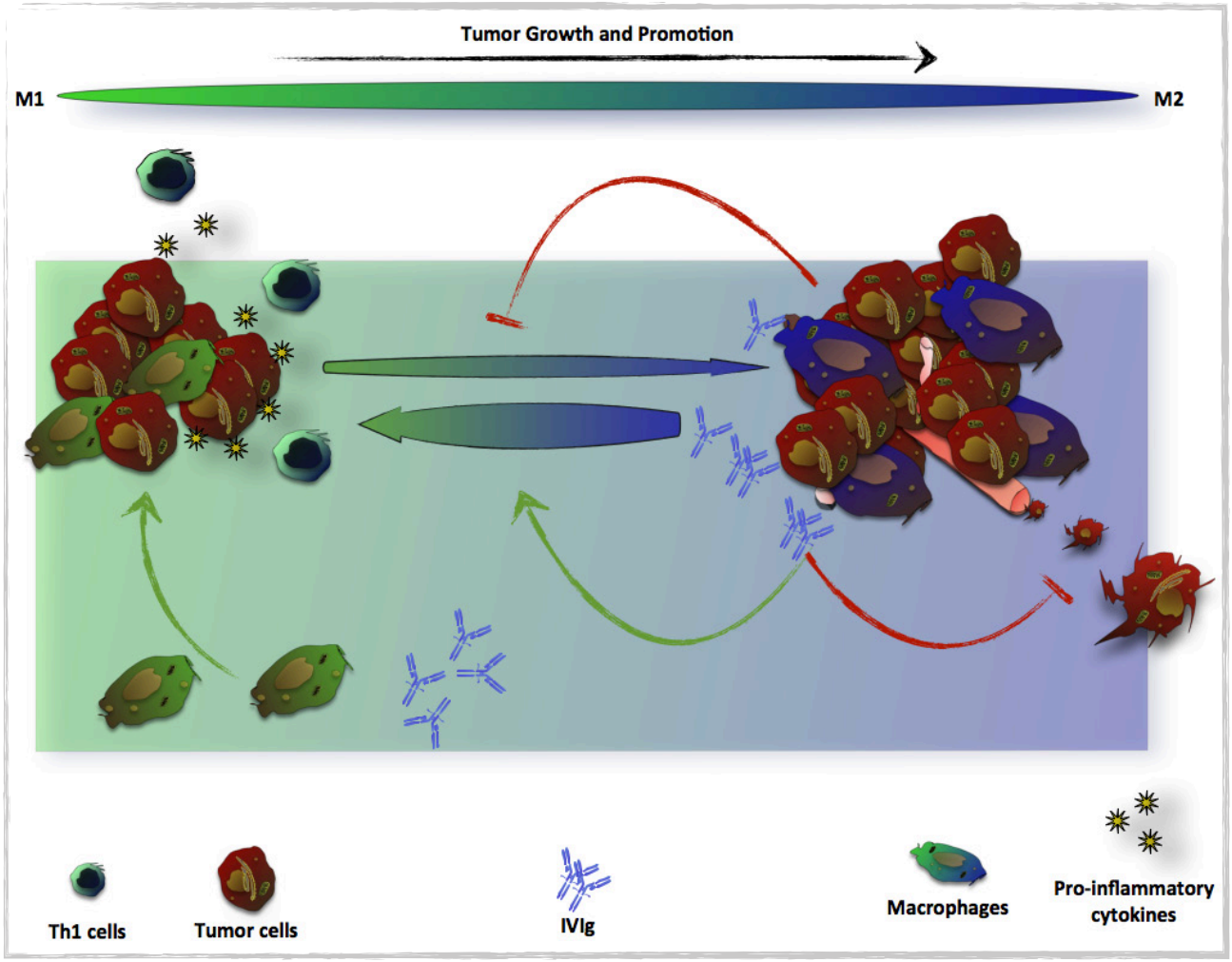


Figure 15. Possible role of IVIg and macrophages anti-tumoral actions.

IL-10 levels in the tumor microenvironment and, therefore, limit the immunosuppressive status of the tumor tissue. Regarding the potential correlation with serotonin, we have observed that IVIg promotes a decrease on *HTR2B* mRNA levels. Thus, we can also speculate the serotonin-driven pro-tumoral effects (e.g., secretion of epiregulin, inhibition of IL12p40 and TNFα) would be limited in the presence of IVIg. Interestingly, we also observed that M1-marker genes, such as INHBA (71), EGLN3 (72) or MMP12, were induced in M2 macrophages upon IVIg treatment. Activin A (INHBA) exerts multiple actions, including cancer cell growth arrest and M1-polarization. Thus, higher levels of activin A (and EGLN3, which has been described to be activin A-dependent (72)) in the tumor microenvironment could help in skewing macrophages towards the M1 anti-tumoral status. Finally, MMP12 up-regulation by IVIg would also result in inhibition of angiogenesis and subsequent reduction of tumor growth and metastasis, since this metalloproteinase has been directly involved in the production of the potent anti-angiogenic molecule angiostatin (265).

Altogether, our results indicate that IVIg could be considered as a potential immunotherapy agent against tumor promotion and metastasis, as it triggers anti-tumoral actions by switching the phenotype and effector functions of TAMs into an anti-tumoral polarization state.

CONCLUSIONS

1. The serotonin (5-HT) receptors 5-HTR_{2B} and 5-HTR_{7} are preferentially expressed by *in vitro* M-CSF-derived macrophages.
2. The receptor 5-HTR_{2B} has been identified on human macrophages *in vivo,* including Kupffer cells and Tumor Associated Macrophages (TAM).
3. 5-HT is able to modulate macrophage polarization towards to an M2-like phenotype through both 5-HTR_{2B} and 5-HTR_{7}.
4. 5-HT and the 5-HTR_{2B} specific agonist BW723C86 modulate the gene expression profile of M-CSF-conditioned (M2) macrophages.
5. 5-HT modifies the macrophage gene expression profile mostly through the 5-HTR_{7}/cAMP/PKA axis.
6. Activation of 5-HTR_{2B} by BW723C86 regulates the expression of type I IFN-regulated genes.
7. 5-HT inhibits the LPS-induced secretion of pro-inflammatory cytokines from macrophages via ligation of 5-HTR_{7}.
8. IVIg reduces the LPS-induced secretion of pro-inflammatory cytokines from M1 macrophages without significantly affecting the transcriptomic polarization state.
9. IVIg switches the phenotypic and functional polarization of human and murine M2 macrophages towards the acquisition of an M1-like state.
10. The IVIg-induced M2-to-M1 polarization switch is partly CD16-dependent in human macrophages, and Fc receptor-mediated in murine macrophages.
11. IVIg exerts an anti-tumoral activity in murine models of cancer, an effect that is dependent on macrophages
12. IVIg treatment triggers an increase in pro-inflammatory gene markers in intra-tumoral macrophages, as well as a change in the basal level of cytokines in the peripheral blood of tumor-bearing animals.

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